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EFFECTS OF INORGANIC SALTS ON RUMEN VOLATILE FATTY ACID PRODUCTION AND PERCENT FAT IN MILK

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VERNON GARNET MACKAY

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ABSTRACT

A simple quantitative method was developed to separate acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids in rumen fluid by gas-liquid chromatography with a column of 10 percent 12-hydroxy-stearic acid on Fluoropak 80. The separated volatile fatty acids were determined in amounts as low as 0.01 mmole per 100 ml of rumen fluid with a flame ionization detector.

Experiments were conducted to study the effects of natural well waters of high mineral salts content and the addition of mineral salts to city water on the production and molar proportions of volatile fatty acids *in vitro*; on the concentrations and proportions of volatile fatty acids in the rumen; and on the percent fat in milk.

No measurable effects were observed in the percent milk fat of lactating Jersey cows fed a natural well water of high soda content, a water high in bicarbonates, a water high in carbonates or demineralized water. The addition of silage to the rations containing the test waters had no apparent effect on the percent fat in the milk. The molar proportions of the volatile fatty acids in rumen fluid were not appreciably altered by the addition of test waters or silage to the ration.

When compared in rumen fermentations *in vitro*, zinc and cobalt salts were associated with a decrease in the production of acetate and n-butyrate commensurate with an increase in the production of propionate and n-valerate versus that of the control or in the presence of sodium, potassium, calcium or magnesium salts. Eight natural well waters, of high mineral salts content, incorporated into the *in vitro* system were similar to distilled water in their effect on volatile fatty acid production, although a trend to a higher production of n-butyrate was observed in the presence of distilled water.

In a lactating cow, sodium bicarbonate, calcium salts or sodium plus potassium phosphates in the drinking water were associated with significantly increased ($P < .05$) concentrations of the volatile fatty acids in the rumen fluid. In a dry cow, the concentrations of volatile fatty acids tended to increase in the presence of sodium or magnesium salts, but the increases were not significant ($P > .05$).

Sodium bicarbonate, calcium salts, sodium plus potassium phosphates, sodium salts or magnesium salts were associated with an increased volume of water, a lower percent of dry matter, greater quantities of volatile fatty acids and a lower pH in the rumen than during the respective control regimens. The relative rates of volatile fatty acid production tended to be greater in the presence of minerals, but significance was not attained ($P > .05$). In general mineral salts had little effect on the molar proportions of volatile fatty acids. The sodium plus potassium phosphates regimen was associated with a slightly lower percentage of propionate and a small increase in the percentage of n-butyrate. At a level of 710 parts per million, zinc or cobalt salts caused anorexia in the cows. The marked depression of the acetate to propionate ratio observed *in vitro* in the presence of zinc or cobalt was not observed *in vivo*.

Water consumption was substantially increased in the dry cow, but not in the lactating cow, when minerals were added to the drinking water. In the lactating cow the percent of milk fat was increased on the sodium plus potassium phosphates regimen and slightly reduced on the zinc salts regimen.

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INTRODUCTION

Under current Provincial regulations[1] milk "shall contain not less than 3.25 percent of milk fat ...." Low fat tests under conditions of winter feeding constitute a serious problem in a number of dairy cattle herds in the Edmonton milk shed. The seriousness of the problem is illustrated by a statement[2] from one of the local dairies that 32 out of the 80 milk producers supplying the dairy had average milk fat tests of 3.2% or lower during the months of March and April, 1962. A conservative estimate[2] of the "low-test" shippers from the largest dairy in Edmonton during the period January to May, 1962, was 15% of the total number of shippers.

From results of a survey of feeding and management practices made in 1958[3] the problem appeared to be most serious on farms where: (1) the drinking water offered to the cattle contained a high level of inorganic salts, and (2) no silage was fed.

Well waters of high total salts and soda content as found in the Edmonton milk shed are slightly alkaline owing to a concentration of "bicarbonates of soda, lime and magnesium" in excess of 700 parts per million. It was reasoned that large intakes of such waters might modify the metabolism of the microbial population of the rumen. If so, any changes would likely be reflected in the production of the steam volatile fatty acids normally found in the rumen. Experimental work as reported in the literature indicates that if the levels of acetic and n-butyric acids are decreased with a commensurate increase in propionic acid, a reduction in

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[1]Alberta Regulations, Provincial Board of Health Regulations, The Public Health Act, Division 9, Section 9-1-2.

[2]Personal communications (anonymity requested) with local dairies. 1962.

[3]Bowstead, J. E. 1958. Unpublished data.

the percent milk fat will probably occur. Further it was reasoned that the inclusion of silage in the ration would: (1) have a neutralizing effect on the alkaline water thus tending to counteract any effect due to a higher pH, and (2) contribute acetic, n-butyric, and other acids which, when absorbed, would serve as precursors of milk fat.

Experimental work was needed to evaluate the effect of the waters and certain minerals on the metabolism of the rumen and the production of low-fat milk by cattle consuming such materials. The artificial rumen approach appeared to offer a means whereby natural and simulated test waters could be screened for their effect on metabolism in the rumen.

In 1959 when experimental work on this project was being outlined the determination of volatile fatty acids was carried out predominately by liquid-liquid chromatographic methods. Such methods have inherent features which limit their usefulness for purposes of the present investigation. It was considered essential to the success of the project that a simple, precise and accurate method for the determination of the volatile fatty acids be available. The relatively new technique of gas-liquid chromatography appeared to offer positive possibilities because of its sensitivity, speed and simplicity, and the refined separations possible. However, the determination of volatile fatty acids in biological fluids, e.g. rumen fluid, was complicated by the presence of water and low concentrations of the acids in question.

REVIEW OF LITERATURE

Gas-Liquid Chromatography

The technique of gas-liquid chromatography (GLC) involves the introduction of a small sample of a chemical mixture, capable of being volatilized, onto a column containing an inert support coated with a specific chemical stationary phase. The column, which has a constant flow of an inert gas passing into it, is maintained at an optimal operating temperature for the specific analysis. The sample components, in the form of a vapor, distribute themselves between the gas phase and the stationary liquid. Differences in the affinity of the stationary liquid for the various component vapors cause them to move at different speeds down the length of the column. The resolved substances emerge from the column as individual "bands" which, upon passing through a suitable detector, give rise to a signal which is usually amplified and fed into an automatic recording device.

The use of GLC was first envisioned by Martin and Synge in 1941 while investigating the concept of liquid-liquid chromatography. However, the practical application of this theory was not published until 1952, when James and Martin (1952) reported the separation of volatile fatty acids (VFA) from formic to dodecanoic by GLC with a column made up of a diatomaceous earth, coated with silicone grease mixed with stearic acid. Nitrogen was the carrier gas, and an integral detection method, which utilized an automatic titration device, was used in this first work.

Since the initial separation of a mixture of VFA by a GLC method in 1952 by James and Martin several methods involving GLC procedures have been used by various investigators to resolve the mixtures of VFA found in biological fluids (el-Shazly, 1952; Annison, 1954a, b, c; Johns, 1953, 1955; Van de Kamer et al., 1955; Armstrong, et al., 1957; Hawke, 1957;

Hankinson et al., 1958; Jamieson, 1959; Raupp, 1959; Smith, 1959; Ellison et al., 1960; Hunter et al., 1960a, b; Vorbeck et al., 1960; Emery and Koerner, 1961; Erwin et al., 1961; Gehrke and Lamkin, 1961; Prevot and Cabeza, 1961; Moore et al., 1962).

In general, procedures evolved for the gas-liquid chromatographic separation of VFA have been governed by the choice of inert support, the stationary liquid phase, and the means of detection of the resolved acids passing from the column. Once these choices have been made the VFA must then be: (1) in a suitable solvent, (2) in concentrations adequate for quantitative detection, and (3) suitable chemical entities (e.g. free fatty acids or esterified fatty acids) for resolution in the specific column selected. The preparation of biological materials preparatory to GLC analysis for VFA has frequently proven to be very tedious and laborious, often fraught with error, and thus the limiting part of the procedure.

Detectors. An integral detection method, which utilized an automatic titration device, was employed by James and Martin (1952) in the original GLC work and later by el-Shazly (1952), Johns (1953, 1955), Annison (1954a, b, c), Van de Kamer et al. (1955), Armstrong et al. (1957) Hankinson et al. (1958), Jamieson (1959), and Hughes (1960).

Another type of detection device, the gas-density balance, was developed by Martin and James (1956). This sensitive instrument made possible the measurement of the true density differences between the pure reference carrier gas and a mixture of the column effluent. As a bulk property detection device, it was linear over a wide range; the degree of signal emitted was related simply to the molecular weight of the component band. Using this instrument James and Martin (1956) carried out the separation of micro amounts of methyl esters of saturated fatty acids from C_1 to C_6. The early version of the gas-density balance was difficult to produce commercially, for technical reasons, and it was not sensitive

enough for high resolution columns (Lipsky and Landowne, 1961).

Prior to 1960 the most commonly employed detectors were bulk property-sensing devices based on a comparison of the changes in the thermal conductivity of a pure reference gas and a gas containing a sample vapor. Although suitable for a number of routine analyses, this form of detector, consisting of either a hot filament or a thermistor, is susceptible to small changes in gas flow, pressure, and temperature. Baseline drifts are a common occurrence and often make the detection of trace components difficult. Since the best devices based on this principle have sensitivities usually no better than that of the gas-density balance, relatively large sample sizes are required. This in turn frequently leads to a decrease in the resolving power of the chromatographic column and prevents the use of smaller concentrations of stationary liquid on the inert support. Further increases in sensitivity are not likely to be attained by these instruments, since they suffer from the disadvantage of measuring a small quantity of vapor that is diluted manyfold by the carrier gas stream (Lipsky and Landowne, 1961). Quantitative estimates of VFA using thermal conductivity detectors were obtained by Hawke (1957), Raupp (1959), Smith (1959), Ellison et al. (1960), Hunter (1960a), Metcalfe (1960), Vorbeck et al. (1960), Gehrke and Lamkin (1961), Prevot and Cabeza (1961), and Moore et al. (1962).

Detectors which have substantial increments in sensitivity over thermal conductivity and which largely avoid the dilution effect on the concentration of sample vapor as it passes through the detector are based on different types of ionization processes. Several detectors of this type are discussed by Johns (1959) and Lipsky and Landowne (1961). Of this type, the flame ionization detector has been used by Emery and Koerner (1961) and Erwin et al. (1961) to measure VFA directly in acidified biological solutions. Detection is based on the premise that very few ions are formed when a flame

of pure hydrogen is burned in air or oxygen. However, when a carbon compound is introduced into the flame, many ions are produced. The flame ionization detector is insensitive to water. The response of this detector is proportional to the carbon content of the compounds and to the stereochemistry of the molecule. The latter is shown by differences in response between iso- and normal-acids (Emery and Koerner, 1961). This detector gives excellent sensitivity permitting measurement of components in the parts per billion concentration range. This is approximately 1000 times the sensitivity of conventional thermal conductivity detectors.

Hunter, Ng and Pence (1960b) used a chromatographic unit employing an ionization detection cell containing 56 microcuries of radium-226 to measure VFA in anhydrous extracts. Argon was the carrier gas. When argon is the carrier gas it can be excited to its metastable state, resulting in a low background current in the absence of sample. Detection depends upon the ionization of organic molecules by collision with metastable argon atoms. The ionization potential of the organic molecule is lower than the excitation potential of the metastable argon atom (11.6 ev). Consequently, there is a transfer of energy from the highly excited but unionized metastable atoms to the organic vapor molecules, which become ionized. The secondary electrons that are produced in the process, when collected at the anode, give rise to an increase in the ionization current, and the increase is related to the vapor concentration. With argon as the carrier gas, the sensitivity is of the order of 300 times that of a thermal conductivity detector (Johns, 1959; Lipsky and Landowne, 1961).

Inert supports. Aggregates of diatomaceous earth (e.g. Celite, firebrick, Chromosorb, Gas-Chrom) were used in the original work of James and Martin (1952) and in most of the GLC work on VFA reported since that time. Prevot and Cabeza (1961) used a teflon powder (Fluoropak 80) as the

inert support when the sample contained considerable water. This avoided the tailing of the water peak into the subsequent VFA peaks as happens when diatomaceous earth supports are used. Water is not absorbed by the Fluoropak 80 and a sharp nearly symmetrical peak evolves on the chromatograms. Moore et al. (1962) used micro glass beads (150-250 μ diameter) as the inert support.

Stationary phases. Anhydrous mixtures of the VFA were originally separated (James and Martin, 1952) on a stationary liquid phase of a mixture of silicone grease and stearic acid or a mixture of silicone grease, stearic acid and H_3PO_4. Numerous other workers have used these phases to resolve anhydrous VFA mixtures or anhydrous ether solutions of the VFA mixtures (Annison, 1954a, b, c; Armstrong et al., 1957; el-Shazly, 1952; Gehrke and Lamkin, 1961; Hankinson et al., 1958; Hughes, 1960; Jamieson, 1959; Johns, 1953, 1955; and Van de Kamer et al., 1955).

Hawke (1957) used a stationary liquid phase of a silicone + behenic acid + H_3PO_4 mixture and Metcalfe (1960) used a diethylene glycol adipate polyester + H_3PO_4 mixture to separate anhydrous diethyl ether solutions of the VFA. Hunter et al. (1960b) separated anhydrous acetone solutions of the VFA on a liquid phase of diethylene glycol adipate polymers.

Hunter et al. (1960a) separated mixtures of the VFA in the presence of water, in amounts up to 50% of the mixture, on a liquid phase of diethylene glycol adipate polymer. Moore et al. (1962) separated aqueous solutions of mixtures of the VFA with a stearic acid + H_3PO_4 phase. However, these two groups of workers placed oxidation and drying chambers before the thermal conductivity detectors to avoid the interference of water.

Smith (1959) using a stationary liquid phase of Tween 80 was able to separate a mixture of VFA containing small amounts of water. Prevot and Cabeza (1961), using a stationary liquid phase of either stearic acid or 12-hydroxystearic acid on Fluoropak 80, were able to separate mixtures

of the VFA which contained up to 80% of water. Because thermal conductivity detectors were used, both Smith (1959) and Prevot and Cabeza (1961) could estimate the quantity of water in the sample.

Emery and Koerner (1961) using a Tween 80 stationary liquid phase and Erwin et al. (1961) with a Tween 80 + H_3PO_4 mixture as the liquid phase were able to separate trace levels of the VFA in aqueous solutions. The VFA were detected with a sensitive flame ionization detector.

The methyl esters of the VFA were successfully separated on stationary liquid phases of dioctyl phthalate or liquid parraffin at 78 C and benzyldiphenyl or paraffin wax at 100 C (James and Martin, 1956). Ellison et al. (1960) separated the methyl esters of the VFA with a stationary phase of silicone oil or diisodecyl phthalate. Vorbeck et al. (1960) used a stationary liquid phase of polyester butanediol succinate to separate the methyl esters of the VFA.

Sample preparation for GLC analyses. The quantitative separation of VFA by GLC offers advantages over older methods because of its sensitivity, speed, and simplicity, and the refined separations possible. However, many investigators working with biological materials have had to overcome certain obstacles before the advantages of GLC were realized. First, the sample introduced onto the chromatographic column must contain a concentration of acids sufficiently high to produce peaks large enough for a good quantitative analysis. Obviously the concentration requirements will vary with the particular chromatographic detector available. Also, if the solid support has an affinity for water, the sample should be anhydrous, especially for the chromatography of free acids, since water gives a very poor chromatographic peak which comes over quickly and tails badly into subsequent peaks. Gehrke and Lamkin (1961) contend that even traces of water tend to upset the chromatographic separation of the acids, making frequent replacement of columns necessary and casting doubt on all results.

However, Hunter et al. (1960a) suggest that water admixed with the acids does not adversely affect the separation in the column.

The direct analyses of VFA in aqueous mixtures has been reported by several groups of investigators. Hunter et al. (1960a) and Moore et al. (1962) oxidized the eluted acids to carbon dioxide and removed all traces of water by placing oxidation and drying chambers, respectively, between the column exit and the detector. In both instances the carbon dioxide was measured in thermal conductivity cells.

Hunter et al. (1960a) reported that solutions containing up to 50% water and the alkali salts of VFA, extracted from biological materials, were chromatographed by GLC after acidification to pH 2 with 85% H_3PO_4. Moore et al. (1962) analyzed the proportions of VFA in the reacidified distillate following the determination of total VFA by steam distillation and direct titration with base. Prevot and Cabeza (1961) were able to separate and quantitatively estimate directly by GLC the water and fatty acids in the aqueous mixture obtained following the oxidation of oleic acid.

Erwin et al. (1961) determined the VFA in rumen fluid and blood. Strained rumen fluid was acidified with HPO_3, allowed to stand 30 minutes and centrifuged at 3000 rev/min for 10 minutes. The supernatant was analyzed by GLC without further preparation. Blood was deproteinized, the filtrate lyophilized after the sodium salts of the fatty acids were formed, the salts acidified, and the solution analyzed by GLC without further preparation.

Most reports dealing with GLC analysis of VFA in biological materials, and in particular rumen fluid, cite methods that employ a steam distillation or solvent extraction procedure. With steam distillation a quantitative determination of the total VFA content may be obtained by direct titration of the distillate with standard base. In any case the salts of the VFA are dried, acidified and taken up in an anhydrous organic

solvent for analysis of the proportions and/or quantities of the VFA.

Several procedures have been used for the esterification of the VFA. Stoffel et al. (1959) prepared methyl esters of the VFA by an interesterification procedure with methanol and hydrochloric acid. Ellison et al. (1960) prepared methyl esters of the VFA in ether solution by adding an excess of diazomethane.

Volatile Fatty Acids in Ruminants

Tappeiner in 1883 (cited by Annison and Lewis, 1959) demonstrated that the fermentation of cellulose in the rumen of the ox resulted in the formation of large amounts of VFA. Elsden (1946) provided the first accurate analyses of the VFA in rumen contents. Until that time little was known about the production, absorption or metabolism of the individual acids. It has been established that the volatile acids which are produced in the digestive tract of the ruminant represent an important source of energy to the host (Carroll and Hungate, 1954; Emery et al., 1956; Annison et al., 1957; Bensadoun et al., 1962).

The amounts and proportions of the VFA produced in the rumen are variable, depending on the nature of the diet, plane of nutrition, time after feeding and age of the animal (Stewart et al., 1958; Barnett and Reid, 1961; Rook, 1961; Baldwin, 1962). Shaw et al. (1959) and Shaw (1959) have suggested that the analysis of rumen fluid for VFA may be a valuable guide for the evaluation of dietary regimens for their probable effect on the fat content of milk and to select rations which might be expected to be the most efficient for fattening purposes. Stewart et al. (1958) contend that the concentrations of VFA in the reticulo-rumen at various times after feeding do not necessarily represent their daily production. Any changes in the volume of rumen contents, rate of production or rate of absorption would be reflected in a change in concentration of the metabolite under study. Unless there is a correlation between the concentration of VFA

in rumen fluid and their total production, information on concentrations adds little to our knowledge of their contribution to the nutrition of the ruminant. The Cornell workers found that whereas only slight differences occurred in the average concentrations of VFA in the rumen between the four rations studied, the total amounts of VFA produced were obviously different. Grieve (1962) found that concentrations of VFA in rumen fluid did not reflect the differences found in the rate of gain and feed intake of sheep fed pelleted and non-pelleted rations.

The VFA presumably are the end-products of digestion in the reticulo-rumen, and the concentrations in the abomasum are low (Phillipson and McAnally, 1942; Gray et al., 1954; Annison, 1954c; Ward et al., 1961). The best estimates of the effects of different dietary regimens would appear to be obtained from studies on the total production of the VFA in the reticulo-rumen and/or the absorption of the VFA into the portal blood from the fore-stomachs of the ruminant.

Techniques for measuring the total contribution of the VFA to the energy economy of the ruminant. Since it was first demonstrated that the VFA are absorbed from the fore-stomachs of the ruminant, a number of workers have attempted to measure the rates at which the individual acids are produced in or are absorbed from the rumen, and on this basis to decide the total amounts available to the animal.

Many attempts to examine the absorption rates directly have involved the introduction of mixtures of the VFA into the rumen of animals maintained in an altered physiological state (Danielli et al., 1945; Gray, 1947, 1948; Masson and Phillipson, 1951; Kiddle et al., 1951; Pfander and Phillipson, 1953; Johnson, 1951; Sutton et al., 1962). While much knowledge has been gained concerning the mechanisms of absorption and the factors influencing absorption, the conflicting results undoubtedly reflect the different

physiological conditions under which the observations were made. In the above mentioned researches the rate of disappearance of VFA added to the rumen was used as a measure of their absorption.

Annison et al. (1957) made a direct assessment of the amounts of VFA which are absorbed from the rumen by a study of differences in the carotid and portal blood. Bensadoun et al. (1962) calculated the total amounts of the VFA absorbed, by sheep fed chopped or pelleted hay, from portal-arterial differences and the measurement of the portal blood-flow rate. The measurement of absorption of VFA in animals under nearly normal physiological conditions (Bensadoun et al., 1962) has provided the first accurate estimates of the quantities of VFA absorbed into the portal blood. These data unconditionally refute earlier reports (McCarthy et al., 1958; Brown et al., 1960) that the relative proportions of VFA found in the portal blood are similar to those produced in the rumen.

The composition of a mixture of VFA undergoes considerable modification in its passage through the rumen wall tissues because the fatty acids are partially metabolized by the epithelium; the extent of change is least for acetate, and greatest for n-butyrate (Pennington, 1952). Conversely, Bensadoun et al. (1962) found that formic acid was present in very small proportions in the rumen liquid (less than 1% of the VFA), but the proportion of formic acid in the portal blood was found to be as high as 35% of the VFA. Therefore, in order to obtain a true rate of absorption of VFA from the rumen, one must ascertain the rate of disappearance of VFA from the rumen rather than their appearance in the portal blood.

Ward et al. (1961) measured the VFA concentrations and proportions in the rumen, abomasum, small intestine, caecum and colon of full-fed beef heifers at slaughter. Substantial amounts of formic and lactic acids were found in the abomasum and small intestine along with acetic, propionic, and butyric acids. Slight quantities of formic and substantial

amounts of lactic acid were also found in the caecum and colon. Millimoles of VFA/100 ml of sample in the rumen, abomasum, small intestine, caecum, and colon were respectively, 11.9, 1.3, 1.9, 12.2, and 11.2. Annison (1954c) examined the VFA of various regions of the digestive tract and found that only the rumen and caecum contained appreciable quantities. The concentration of VFA in the caecum was about one-quarter of that in the rumen. Annison (1954c) concluded that since the caecum has only about one-tenth the capacity of the rumen it is probable that the contribution of VFA from the caecum is small.

Since the portal system drains the whole splanchnic area, estimates of VFA production derived from portal blood concentrations contain the VFA produced in regions of the gastrointestinal tract other than the rumen. Bensadoun et al. (1962) have not taken this into consideration when comparing differences in relative proportions of the VFA in the rumen and in the portal blood. Similarly, other investigators who have estimated total VFA production from rumen production of these acids have neglected the possible production of VFA in other parts of the digestive tract.

Another approach to the problem by several investigators (Carroll and Hungate, 1954; Halse and Velle, 1956; Stewart et al., 1958; Hungate et al., 1961) involves transfer of whole rumen contents to fermentation vessels and conduction of fermentations in vitro. A curve is constructed from the increase in VFA levels in the sample during the incubation period. The slope of the curve at zero time is assumed to be the rate of production in vivo at the time the sample is obtained. Total production of the individual acids can be estimated from the rates of production, determined at various times, and a knowledge of rumen volume and dry matter content. By this procedure Stewart et al. (1958) have, under essentially normal conditions, estimated the production, absorption and energy contribution of the VFA produced in vivo. This can be done since

the VFA are absorbed, and not metabolized further in the digestive tract, and since the concentrations of the VFA in the abomasum are low. Under these conditions the total production of VFA during a 24-hour period should equal the total absorption.

Gray et al. (1960) measured the rates of production of acetic, propionic and butyric acids in the rumen of a sheep from the decline in specific activity of these acids in the rumen following addition of their sodium salts-1-C^{14} to the rumen. Polyethylene glycol was used as a marker to permit measurement of the rumen volume. Sheppard et al. (1959) added acetate-1-C^{14} to a sheep's rumen and measured the rate of acetic acid production by isotope dilution. Davis et al. (1960) and Essig et al. (1961) measured the amount of acetate absorbed from the gastrointestinal tract of a fed animal, using the isotope dilution technique. Acetate-1-C^{14} was infused into the jugular vein until a balance was obtained between the infused, labelled acetate and unlabelled acetate entering the system; i.e. the specific activity remained constant. The decrease in blood acetate specific activity following feeding gave a measure of the amount of acetate absorbed from the fore-stomachs of the ruminant.

McCarthy et al. (1958) and Brown et al. (1960) used a rumen perfusion technique to obtain estimates of the absorption of the individual VFA. The estimates of VFA production were obtained under well defined conditions, such as rumen fatty acid concentration, rumen pH, and rate of blood flow through the rumen vascular system. Such rumen perfusion data should be interpreted in light of the unphysiological conditions which exist, particularly in view of the low blood flows through the rumen vascular system. Bensadoun et al. (1962) concluded that the discrepancy between the results of rumen perfusion studies and their absorption studies in intact, conscious sheep was largely attributable to a difference in the techniques. The isolated rumen appears to have an altered physiological behavior.

Factors affecting the absorption of VFA. The experiments of Danielli et al. (1945) and Gray(1948) demonstrated the significant effect of the pH of the rumen contents on absorption of VFA. When the rumen contents were acid (pH 5.8) the VFA were absorbed at different rates and the relative rates of absorption were butyric>propionic>acetic. At a pH of 7.5 Danielli et al. (1945) found that the rates of absorption of the acids were much lower and the relative rates of absorption were acetic>propionic>butyric. Gray (1948) using a different technique found that there was little or no absorption when the rumen contents were alkaline (pH 8.5). It appears that the rumen is much more permeable to the free acid than the anion. At neutral pH there is a close relationship between the disappearance of fatty acid from the rumen and the appearance of bicarbonate. The rumen is readily permeable to carbon dioxide and it would appear that a supply of hydrogen ions from the conversion of carbon dioxide to bicarbonate allows the transformation of fatty acid anion into the unionized form which is then absorbed. At neutral pH only half the total fatty acid taken up could leave as the free acid because the other half is equivalent to a net movement of strong electrolytes from the rumen, mainly as sodium, and must leave as the anion (Dobson, 1961).

The pH of a test solution, whether alkaline or acid, placed in a washed rumen tends to approach that of blood even if saliva be prevented from entering the rumen (Sutton et al., 1962). The transfer of ions across the rumen wall is assumed to be the cause of the pH changes. Danielli et al. (1945) and Sutton et al. (1962) suggested that the absorption of free acid was a cause for the rise in pH of a test VFA solution introduced into a washed rumen wherein saliva flow was blocked.

The VFA are absorbed at rates depending on their individual concentrations in the rumen (Brown et al., 1960; Stewart et al., 1958; Sutton et al., 1962). Various other factors seem to affect absorption rates of

VFA from the rumen. However, there is a lack of data on this subject, and it is not known whether the factors involved are of a physiological or biochemical nature. Dobson (1961) has summarized several related observations, including some of his own findings, and concluded that treatments which depress fatty acid uptake include cannulation during the course of an experiment, leaving the rumen filled with NaCl solution overnight before the absorption experiment and starvation. The Rowett group has shown that blood flow through the rumen epithelium is dependent on the composition and acidity of the solution in the rumen. Armstrong et al. (1957) suggest that acid absorption is a process which necessitates an actively metabolizing rumen epithelium. Since the rumen epithelium metabolizes n-butyrate, and propionate to a lesser extent, the effects of starvation in reducing its ability to absorb the VFA are understandable.

Sutton et al. (1962) suggest that surgical procedures and/or apparatus (e.g. the balloon of an esophageal catheter) may affect rumen motility and hence absorption rates. Improved techniques which, while allowing reproducible conditions, will allow one to study the factors affecting absorption in an animal under relatively normal conditions are needed.

The effect of minerals on the intra-rumen environment and VFA production. Experimental evidence relating the role of minerals to the metabolic activities of the rumen microorganisms and to the absorption of VFA is inadequate. Some indications of the effects of various minerals on the intra-rumen environment and VFA production have been derived from in vitro studies and from feeding-trial and digestibility experiments. Barnett and Reid (1961) suggest that since the rumen wall may have an active role in selectively absorbing the VFA into the blood stream, it would appear that the rumen epithelium may perform a somewhat similar function with mineral elements. Minerals might be expected, if this be the case, to

influence either the production of VFA within the rumen or the absorption of VFA from the fore-stomachs.

Numerous studies *in vitro* have been conducted to examine the mineral requirements of rumen bacteria. Such criteria as decrease in non-protein nitrogen (McNaught *et al.*, 1950) and cellulose digestion (Hubbert *et al.*, 1958a,b) have been used to establish the optimum and toxic levels of a number of minerals to rumen bacteria. In addition Bryant and co-workers, USDA, Beltsville, Maryland, have published extensively on the mineral and other requirements of pure cultures of ruminal flora.

Burroughs *et al.* (1950) were the first of many groups to observe that when alfalfa hay, or an equivalent amount of alfalfa ash, was added to rations containing a high percentage of corncobs, or poor quality forages, the digestibility of fiber was greatly improved. The alfalfa ash effect has now been generally ascribed to its "trace-mineral" content, e.g. Co, Cu, Mn, Fe, Zn, Mo (Barnett and Reid, 1961). Nicholson *et al.* (1960) studied the influence of mineral supplementation of low ash rations on intra-ruminal environment and the performance of the animal. The addition of alkaline mineral supplements tended to improve feed consumption and rate of gain. When the roughage in the ration was corncobs and timothy hay, the addition of 5% alfalfa ash tended to increase the rumen fluid concentration of total VFA measured at 6 hours after feeding. The percentage of acetic acid tended to be higher and the percentage of propionic acid lower on rations supplemented with alfalfa ash. Rumen pH values tended to be higher on the alfalfa ash-supplemented rations. Also associated with alfalfa ash supplementation of the rations was an increased consumption of water, greater percent of water in rumen ingesta and an increased excretion of urine. Estimates were not obtained on the effects of the alfalfa ash supplementation on the production of VFA.

Nicholson and Cunningham (1961) and Nicholson et al. (1962a, b) added sodium propionate, $NaHCO_3$, K_2CO_3, and $CaCO_3$ at various levels and in several combinations to high and low roughage rations for beef cattle. Growth and feed efficiency results were inconsistent from trial to trial. It was noted that 5.7% limestone in a ground grain ration depressed digestibility of dry matter and nitrogen. Also limestone did not increase urine excretion as did $NaHCO_3$. In more recent experiments Nicholson et al. (1962c) found that the addition of 3% $NaHCO_3$ to all-concentrate rations for steers resulted in a significant increase in food intake. In digestibility trials with fistulated Jersey steers it was found that buffers (3% $NaHCO_3$ or 3% $NaHCO_3$ plus 2% limestone plus 1% K_2CO_3) added to all-concentrate rations caused consistent decreases in percent dry matter, total VFA concentration, molar percent acetic acid and the acetic to butyric acid ratio of the rumen ingesta. The pH and buffering capacity of the rumen fluid from buffered rations were higher than those of the control rations. The buffers caused no significant changes in propionic acid concentrations.

The addition of 0.5% $NaHCO_3$ to the drinking water offered to steer calves fed a pelleted hay-concentrate ration during the winter significantly increased the average daily gain and rumen pH levels from those of the animals fed the pelleted ration without $NaHCO_3$ (Cullison and Ward, 1961). The steers receiving $NaHCO_3$ in the drinking water drank more than did comparable steers receiving plain water. In a summer feeding trial $NaHCO_3$ at levels of 0.25, 0.5, and 0.75% of the drinking water had no effect on average daily gain of two-year-old steers. The authors suggest an interaction between $NaHCO_3$ and environmental temperature and/or age of the animal.

Lassiter et al. (1961), using steers fed pelleted rations, observed highly significant regressions of anaerobic populations and acetic, propionic, butyric and valeric acid concentrations on $NaHCO_3$ consumption and on time.

Lassiter and Cook (1961) found that 0.5% $NaHCO_3$ in the water fed to steers consuming a complete pelleted ration had no significant effect on digestibility. Water intake was significantly increased owing to the addition of $NaHCO_3$.

When Raun and Burroughs (1961) fed a ration containing 85% concentrate and 15% roughage to lambs they found that additions of Na- and $KHCO_3$ (1.0 to 5.0%), $CaCO_3$ (0.6 to 2.5%), and $MgCO_3$ (0.25 to 1.0%) were without effect in altering rumen acetate-propionate ratios and total VFA concentrations. Similar results were obtained in subsequent experiments when $CaCO_3$ was fed at a level of 0.5% and $NaHCO_3$ at levels of 1.5 and 3.0% of 80 concentrate:20 roughage rations. $NaHCO_3$ tended to increase both rumen pH and total VFA concentrations (Raun et al., 1962). It was noted that the buffering capacity of rumen fluid diminished slightly with the addition of dietary $NaHCO_3$; the authors discussed the implication that movement of buffering substances from the blood into the rumen was reduced when $NaHCO_3$ was included in the diet.

Supplementation of pelleted rations with Co (Rhodes and Woods, 1961, 1962; Raun and Burroughs, 1961) increased the level of propionate while lowering the level of butyrate. The percentages of iso-butyric and iso-valeric acids were lowered and the percent of n-valeric acid raised under conditions that narrowed the acetate to propionate ratios (Rhodes and Woods, 1962). Trace mineral supplementation (Mn, I, Co, Fe, Cu, Zn, S) of rations for lambs tended to widen the acetate to propionate ratio (Rhodes and Woods, 1961) whereas in other experiments the trace mineral effect was inconsistent (Rhodes and Woods, 1962).

Chance and Loosli (1961) reported that the addition of a complex mineral-vitamin supplement to the usual rations of lactating cows had no measurable effect upon milk or fat production, efficiency of milk production, or digestibility of the rations.

Oltjen et al. (1962) compared several effects of two complex acid or basic mineral mixtures in the rumen of steers when included in a purified ration containing urea. The acid mineral mixture contained a high proportion of PO_4^{---} and SO_4^{--} as its complement of anions, whereas the main anions in the basic mineral mixture were PO_4^{---} and CO_3^{--}. The steer fed the basic mineral mixture had a higher rumen pH, larger rumen volume, greater total milliequivalents of VFA, and a greater average concentration per gram of dry rumen contents of riboflavin and thiamine. Relative ruminal concentrations of the individual VFA were not affected by the mineral mixtures. When rumen fluid was taken from each steer three hours after feeding and used as an inoculum in an in vitro system it was found that the inoculum from the steer fed the basic mineral mixture digested nine times as much cellulose as did that from the steer fed the acid mineral mixture. The production of VFA either in vivo or in vitro was not reported, but it is interesting to note that these data indicate a greater synthesis of riboflavin and thiamine when the alkaline mineral mixture was fed. The authors speculate that the B-vitamin synthesis with the purified diets was insufficient to promote maximal fermentation and utilization of the VFA.

Matrone et al. (1957, 1959) have found that the addition of Na- and $KHCO_3$ to purified diets, consisting of readily fermentable substances, caused a beneficial effect on growth. These workers suggest that since the diets were readily fermentable, the rumen acids would be formed more quickly than on a normal diet, thus requiring more buffering capacity at a given time. As the animals did not ruminate appreciably when fed the purified diets, the flow of saliva was probably reduced, further decreasing the total buffering capacity of the rumen. The physiological role played by Na- and $KHCO_3$ in the rumen environment may induce shifts in the population of rumen microorganisms, and in quantities and rate of transport of

end products which in turn affect productive energy of the diet (Matrone et al., 1959).

Van Campen and Matrone (1960) studied the incorporation of labelled carbon into the individual VFA when $NaHC^{14}O_3$ was added to two purified diets in which the only difference was the presence or absence of Na- or $KHCO_3$. On both diets it was found that the propionate contained a major portion of the label, followed in order by valerate, butyrate and acetate. The maximum incorporation of labelled carbon in propionate and valerate was reached at one and three hours, respectively, in the sheep fed the diet with added bicarbonates, whereas maximum values for these two acids were observed at one-half hour and two hours, respectively, in the sheep fed the diet containing no bicarbonates. Percentage incorporation of labelled carbon on the diet containing added bicarbonates was more than two-fold that on the diet containing no added bicarbonates. Total VFA concentrations in the rumen of the sheep fed the bicarbonate-free diet were lower and decreased more rapidly with time after feeding than that in the rumen of the sheep fed Na- and $KHCO_3$. In the latter case the level of propionate in the rumen approached, and sometimes exceeded, the level of acetate in the rumen, whereas on the bicarbonate-free diet the level of propionate in the rumen was consistently less than one-half that of acetate.

The results of anaerobic fermentations, using $C^{14}O_2$, of the rumen ingesta from sheep fed purified diets suggest that CO_2 has a major role in the formation of ruminal VFA, mainly as a precursor of propionic acid, and subsequently valeric acid. Similarly, using acetate-2-C^{14}, it was found that acetate is a major precursor of butyrate and valerate. The formation of valerate seems to involve a condensation of a two-carbon unit with a three-carbon unit. All of the above reactions apparently were enhanced by the presence of Na- and $KHCO_3$ in the diet (Van Campen and Matrone, 1960).

The results of Van Campen and Matrone (1960) are at variance with those of Otagaki *et al.* (1955) The latter group found less than 1% of the specific activity in the VFA when $NaHC^{14}O_3$ was incubated with rumen fluid *in vitro* for 48 hours. The purified rations used by Matrone *et al.* (1957, 1959) and Van Campen and Matrone (1960) may enhance the growth of bacteria responsible for propionic acid production. Since the bacteriological examination showed considerable difference in the flora developed on purified rations (Barnett and Reid, 1961) it seems possible that this might explain the divergent results of the two groups.

Emery and Brown (1961) added 1 lb of Na- or $KHCO_3$ per day to the rations of cows fed grain to appetite and 2 lb of long or pelleted hay. With lactating cows the bicarbonates prevented a decline in milk fat percentage. With nonlactating cows bicarbonates were associated with an increased pH of rumen contents, but had no apparent effect on the molar concentrations of VFA in the rumen. Unfortunately the effects of bicarbonates on VFA absorption were not studied during these experiments.

A report from Italy (Bonomi and Cabassi, 1960) indicated that feeding $ZnSO_4$ at the rate of 0.5 mg per kg of body weight daily increased the fat content of milk of cows by 0.31% without affecting milk yield.

Bladen and Doetsch (1959), using a washed cell suspension *in vitro* technique, found that changes in phosphate buffer concentrations affected the amounts and ratios of VFA and lactic acid produced.

Using average concentrations of Ca^{++} and Mg^{++} in the rumen and blood plasma, and a potential difference of about 30 mv (blood positive with respect to rumen contents), Storry (1961) calculated that unless there is a ninefold concentration ratio, absorption as freely diffusing ions will not occur. Active absorption of Ca^{++} and Mg^{++} across the rumen wall may occur but it has not been demonstrated.

Parthasarathy and Phillipson (1953) found that K^+ had no effect on the absorption of acetate or propionate from the rumen, nor was there any evidence to show that fatty acid absorption was affected by either Na^+ or Cl^-.

Gray (1948) and Sutton et al. (1962) studied the effect of inorganic phosphate on the absorption of VFA from solutions placed in an emptied, washed rumen. The addition of inorganic phosphate had no significant effect on the quantities of VFA absorbed other than through its effect on the pH of the rumen concents.

Graf and Holdaway (1952), Blosser and Soni (1957), and Allen et al. (1958) have compared hard water with water softened by an ion-exchange process; no differences were observed in milk or fat production of dairy cows. Neither was there any difference in consumption between the two types of water.

In a change-over experiment, six heifers were supplied drinking water containing 0,1 or 2% added NaCl for 30 days (Weeth et al., 1960). The 1% level of NaCl caused no obvious effects other than a 52.8% increase in water consumption. The 2% level of NaCl was toxic and induced severe anorexia.

Lesperance and Bohman (1962) found that 100 ppm inorganic Mo in the ration of weanling heifers was toxic. The addition of 0.5% SO_4^{--} to the Mo ration appeared to increase toxicity.

Martin et al. (1962) compared the digestibility of cellulose in a medium supplemented with different levels of Mg and/or S by the rumen ingesta obtained from steers fed a purified ration deficient in Mg and/or S. The digestibility of cellulose and the total VFA concentration of the rumen liquor were decreased when the ration was deficient in Mg and/or S.

Relationships between rumen fermentation and milk fat. Metabolizable energy in ruminants is incorporated into body gains of energy with a lower efficiency than when it is used for maintenance (Blaxter, 1960). For most rations, the efficiency with which an animal can use the metabolizable energy for production is usually within the range from 35 to 65 percent. This wide range is attributable to the effects of different feeds and rations on the fermentation and digestive process and, in particular, on the proportions of VFA, lactic acid and monosaccharide which are absorbed. Blaxter (1960) has summarized the findings up to that time and concluded that there seems no indication of synergistic effects within the possible physiological ranges in which the proportions of the end-products of the rumen fermentation might fall. A given quantity of a mixture of VFA in which acetic acid predominates will result in a smaller synthesis of body fat than would an equicaloric mixture in which propionic acid and lactic acid are present in large proportions. Different considerations apply to lactation in ruminants where lipogenesis is in part dependent on a supply of acetic acid (Blaxter, 1960).

Rook (1961) has recently summarized the current knowledge on the biosynthesis of milk fat in the mammary gland of the ruminant. Milk fat arises from two main sources: (1) a portion, containing the long chain fatty acids, is acquired preformed from the plasma "neutral fat", which may be of dietary origin, and (2) a portion is synthesized within the mammary gland from glycerol, formed in liver or mammary gland, and C_4-C_{16} fatty acids which have been synthesized within the mammary gland from acetate and a C_4 molecule which seems to be β-hydroxybutyrate.

The amount of acetate available to the udder may be regulated directly by acetic acid production in the rumen. McCarthy (1961), in a review, concluded that β-hydroxybutyric acid normally arises from hepatic lipid metabolism. Pennington (1952) had shown that when butyrate was

present in large quantities it tended to be ketogenic and contributed to the formation of β-hydroxybutyrate. Rook (1961) suggests a similar effect occurs when blood levels of acetate are high. The amount of β-hydroxybutyrate produced by the liver is controlled indirectly by the amount of fat oxidation occurring in the liver. Since less fat is oxidized when ample amounts of carbohydrates are present this means that less β-hydroxybutyrate will be available when propionate predominates in the rumen. These observations show that a decline in the milk fat content in ruminants could arise as the result of a decrease in the ruminal production of acetate, or an increase in the production of propionate, or both.

The relative proportion of acetic acid in the rumen is positively correlated ($r = +0.64$, $P < 0.01$) to the percent fat in milk, whereas the relative proportion of propionate in the rumen is negatively correlated ($r = -0.63$, $P < 0.01$) to percent fat in milk (Shaw et al., 1959).

A better picture of the effects of the volatile fatty acids produced in the rumen of the cow on milk yield and composition has been provided by Rook (1961) and Rook and Balch (1961). A method for the continuous intraruminal infusion of the VFA was developed and up to 2 kg/day of the individual acids were added as their dilute aqueous solutions. With Friesian cows on diets of 16-18 lb hay per cow and 4 lb concentrate per gallon of milk, additions of acetic acid caused an increase in the yield of milk, and in the yield of all the major milk constituents, and specifically increased the fat percentage. Propionic acid decreased milk fat percentage and increased the protein percentage. Butyric acid increased the fat percentage, and lactic acid increased milk protein percentage.

Work is needed to measure the correlations between the total production of the individual VFA and percent fat in milk and to evaluate the interaction of one acid on another in the metabolism of the intact lactating cow.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

General outline

The experimental work reported in this thesis is divided into four parts. Part I considers the application of a gas-liquid chromatographic technique to the analysis of VFA in rumen fluid. Part II describes an attempt to develop the low milk-fat syndrome in lactating Jersey cattle by adding natural and synthetic high mineral waters to the ration and a study of the effect of the mineral waters and mineral waters plus silage on the molar proportions of VFA in the rumen ingesta. In part III a series of mineral salts and natural well waters were screened and compared for their effects on VFA production *in vitro*. Part IV describes the effect of several mineral salts in the drinking water of fistulated cows on the intra-rumen environment with particular reference to the VFA.

Statistical analyses

Where applicable the functional analysis of variance (F-test) was used to aid in interpreting the data. When a significant value of F was attained, the multiple range test described by Duncan (1955) was employed to discriminate between means which might differ significantly from other means.

I. A Method for the Determination of VFA in Rumen Fluid

Object

To develop a method for the direct quantitative estimation of VFA in rumen fluid utilizing GLC techniques.

Experimental

Preparation of sample. Fresh rumen fluid, strained through six layers of cheese cloth was acidified to a pH of less than 2 by adding 0.3 ml of 50% (v/v) H_2SO_4 to 25 ml of strained rumen fluid in a 90 ml centrifuge tube. The contents of the tube were thoroughly mixed with a glass rod, and after standing for 30 min, centrifuged at 2600 rev/min for 15 minutes. The supernatant was analyzed by gas chromatography without further preparation. Samples not analyzed immediately were stored in a refrigerator at 40 F.

Instrumentation. A commercial gas chromatograph[1] with a thermal conductivity detector was used in the initial stages. Preliminary investigations indicated that this type of instrument lacked sufficient sensitivity to quantitatively detect even the acids present in highest concentration in rumen fluid. It was therefore necessary to replace this instrument with one with more sensitivity.

The instrument[2] used to obtain the results reported in this thesis was equipped with a flame ionization detector. The flash vaporizer temperature was maintained at 200 C to provide rapid vaporization of liquid samples before entering the column. The column, a 2.5 m x 3 mm internal diameter stainless steel tube, was packed with 10% (w/w) 12-hydroxystearic acid on Fluoropak 80[3]. A column temperature of 150 C was used with a helium

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[1]Burrell Kromo-Tog, Model K-2, Burrell Corporation, Pittsburg, Pa.
[2]Burrell Kromo-Tog, Ionization Model K-7, Burrell Corporation, Pittsburg, Pa.
[3]The Fluorocarbon Co., 1206 East Ash Ave., Fullerton, California.

carrier gas flow rate of 55 ml/minute. Hydrogen and air flows to the detector were controlled by filter orifices to approximately 25 ml and 325 ml per min, respectively. A direct current potential of 250 volts was placed from the tip of the burner to the ion collector which was located above the flame. The output of the ionization detector electrometer was fed to a potentiometer recorder[1] with a full scale deflection at 1 millivolt and pen speed of 1 second.

VFA analysis. Three to 4 microliters of sample were introduced into the flash vaporizer with a 10 microliter syringe[2] graduated to 0.1 microliters. The following injection procedure was used to compensate somewhat for a variable amount of sample remaining in the microsyringe after an injection. With the syringe grasped by the flange and plunger, to avoid warming the barrel of the syringe with the hand, the needle was extended into the sample, air was pumped out and the syringe overfilled; the syringe plunger was depressed to the 3 microliter scribe line, the needle lightly blotted and the plunger backed off, pulling the liquid from the needle into the syringe where the total volume could be read against a white background; the needle was inserted through the silicone seal of the flash vaporizer, the liquid discharged and the needle rapidly withdrawn; the plunger was backed off and the initial less the final volume in the syringe was assumed to have been the amount of sample injected.

The free fatty acids, acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric, were eluted from the column as nearly symmetrical peaks in 32 minutes (Figure 1). Under the conditions of the analysis, the separation of iso- and n-butyric acid was incomplete, particularly with

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[1]Model Y143X58-VB-11-111-30V, Minneapolis-Honeywell Regulator Co., Brown Instruments Division, Philadelphia, Pa.

[2]Model 701N, Hamilton Company Incorporated, Whittier, California.

higher concentrations of the iso-acid. The instrumental conditions employed represent a compromise between resolution and retention time; in order to expedite the analysis maximum resolution was sacrificed.

Three known mixtures of the VFA in water, representing the range of concentrations found in rumen fluid, were used to obtain standard curves as shown in Figure 2. The standard solutions were analyzed in duplicate each operating day, at intervals within a series of the unknown samples. Quantitative determination of the individual acids was accomplished by weighing the chart paper circumscribed by the elution peaks. The concentrations of VFA in the unknown solutions were calculated from the data for known solutions. The chromatographic peaks were identified by comparing their retention times with those established for pure fatty acids.

Estimates of the precision of the method, for each of the acids, were calculated (Table 1) from the results obtained with the standard solutions during 14 operating days within an 18-day period. The rumen liquid samples of part IV were analyzed at the same time. A proof-addition trial gave a measure of the recovery of acetic, propionic, n-butyric and n-valeric acids from rumen fluids diluted 50% with aqueous VFA standard solutions (Table 2). Similar calculations were not carried out for iso-butyric and iso-valeric acids as they were usually present in such low concentrations that small absolute differences were unrealistic when expressed in percentages.

Several column preparations were tested in efforts to improve the resolution of the VFA and/or reduce the time required to elute the higher VFA. A linear increase in the column temperature per unit of time during the analysis was studied in an attempt to give improved resolution of iso- and n-valeric acids.

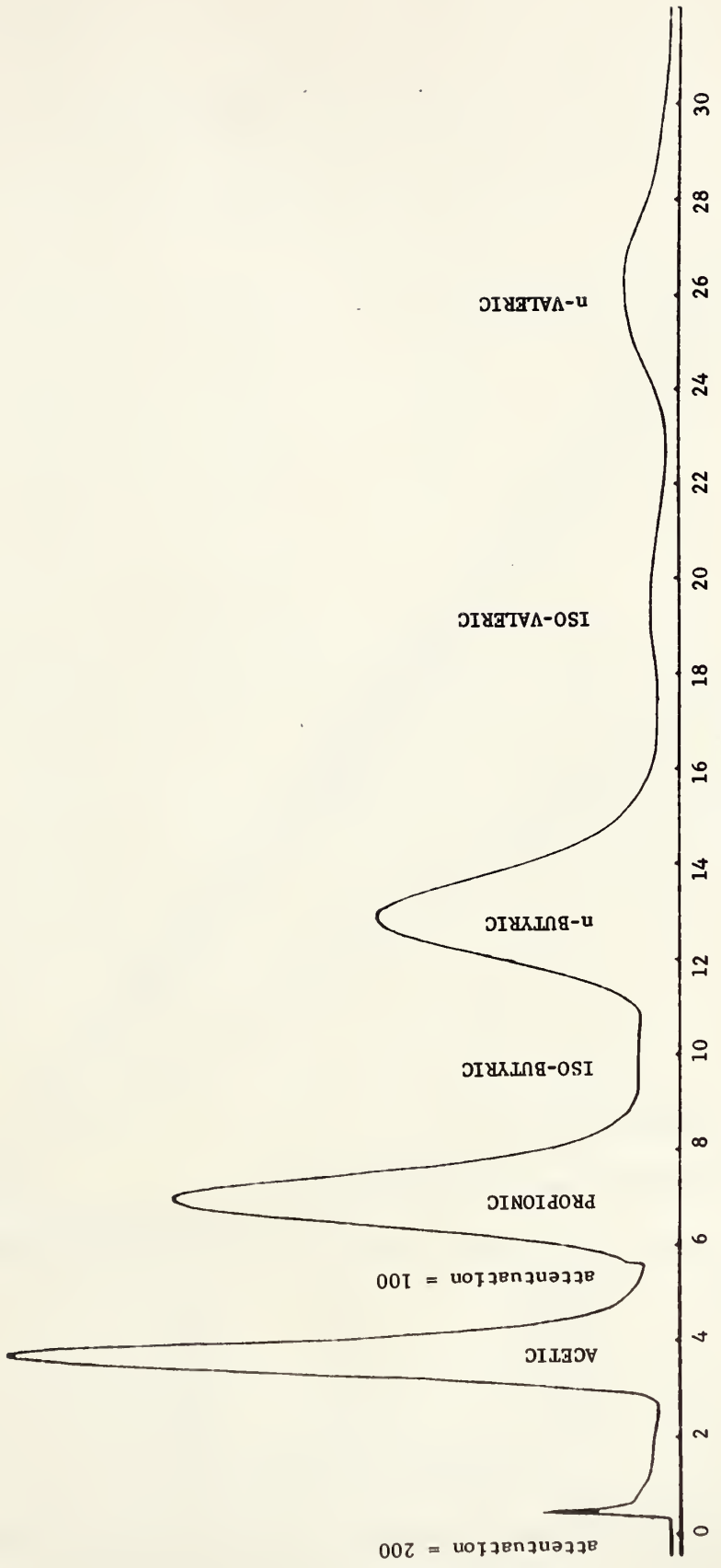

Figure 1. Typical chromatogram of volatile fatty acids in rumen fluid.

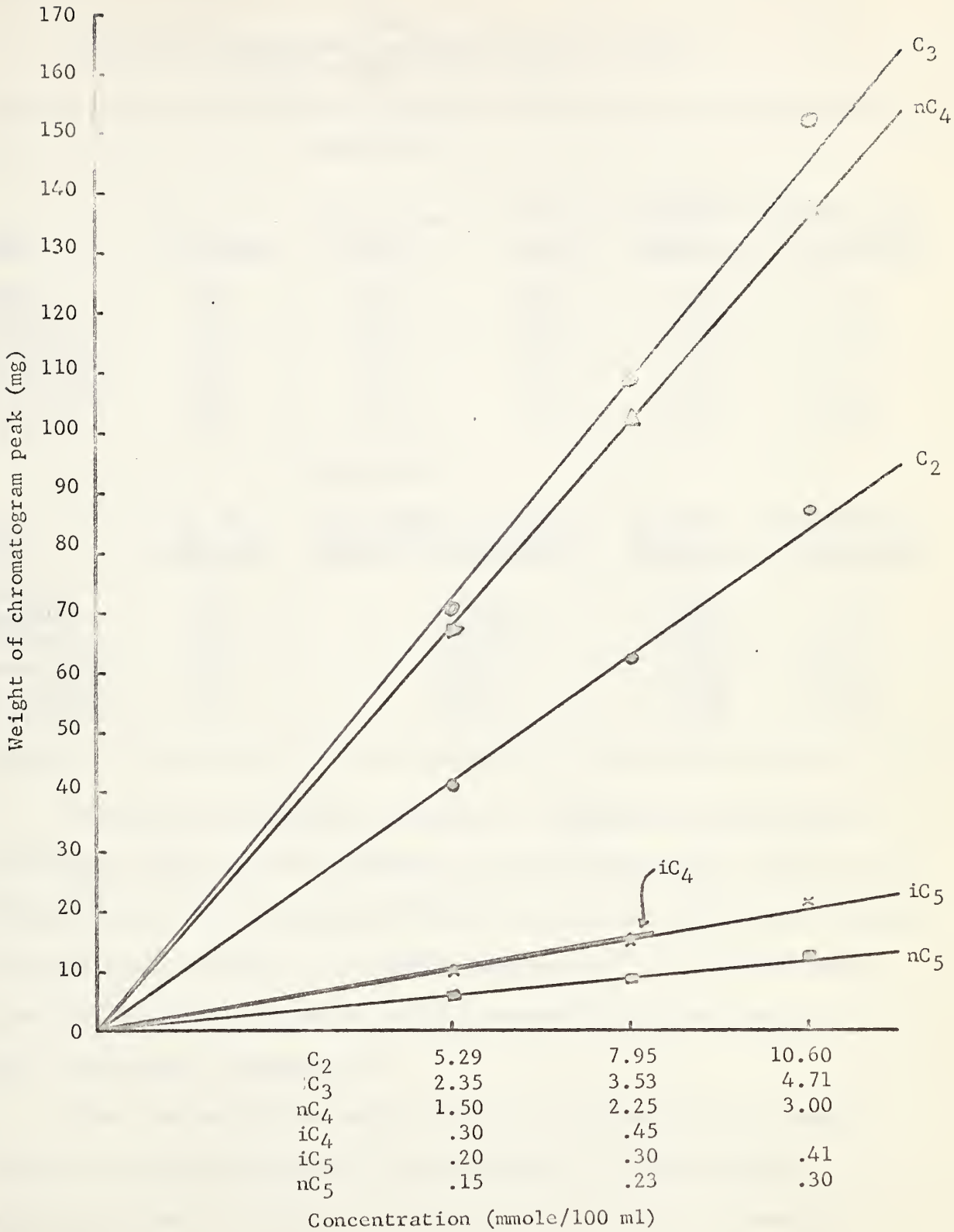

Figure 2. Peak weight as a function of concentration of VFA. (C_2 = acetate; C_3 = propionate; nC_4 = n-butyrate; iC_4 = iso-butyrate; iC_5 = iso-valerate; nC_5 = n-valerate)

Results and Discussion

Table 1
Precision of qualitative and quantitative analysis
of standard solutions of VFA

Qualitative

Acid	No. of analyses	Calculated mole %	GLC Mole %	GLC Standard deviation	GLC Coefficient of variation
acetic	28	54.0	53.8	0.6	1.13
propionic	28	24.0	24.0	0.4	1.60
n-butyric	28	15.3	15.5	0.3	1.79
iso-butyric	28	3.1	3.1	0.1	4.77
iso-valeric	28	2.1	2.0	0.2	8.10
n-valeric	28	1.5	1.6	0.2	9.88

Quantitative

Acid	No. of analyses	Avg detector response mmole/100 ml/mg paper	Standard deviation	Coefficient of variation
acetic	84	.1259	.0054	4.29
propionic	84	.03220	.00144	4.47
n-butyric	84	.02203	.00088	3.99
iso-butyric	56	.02874	.00160	5.57
iso-valeric	84	.01973	.00191	9.68
n-valeric	84	.02518	.00296	11.76

The data for precision of analysis of standard solutions (Table 1) and recovery from rumen fluid (Table 2) are very encouraging. Qualitatively the separations of VFA in aqueous solutions are simple, precise and accurate, but quantitative estimates are somewhat less precise. This is illustrated by the coefficients of variation and the standard deviations shown in Table 1 and Table 2, respectively.

Since the qualitative aspects of the separation of VFA in aqueous solutions are very satisfactory, it is reasonable to assume that the sampling technique is suspect if the quantitative results are somewhat lacking in precision. Under the instrumental conditions employed, the size of sample which would allow optimum resolution of all acids in the sample and highest sensitivity combined with a steady baseline was 3 to

4 microliters. The microsyringe had a 2-inch stainless steel needle cemented to the glass barrel of the syringe. Several syringes of this type were found to have dead volumes ranging from 0.9 to 1.3 microliters. When the needle of the microsyringe was extended through the silicone seal into the flash vaporizer the amount of sample remaining in the syringe was always less than the dead volume of the syringe due to the vaporization of the sample caused by the heating of the needle. The amount of vaporization and loss from the needle, either in the flash vaporizer or immediately after withdrawal, was somewhat dependent upon the injection technique.

With the naked eye the volume in the microsyringe could be read accurately to the nearest 0.1 microliter and estimated to the nearest .05 microliter. It is conceivable that an error of $\pm$ 0.1 microliter could arise on one injection by the procedure employed. Considerable difficulty was had in avoiding parallax errors while making the readings.

Once improvements are made in the precision with which a sample can be injected onto the column, the chromatography of VFA in aqueous solutions will undoubtedly gain much in popularity for the quantitative determination of VFA in rumen fluid and other biological materials. With the instrumentation used in the present experiments much improvement in sample injection accuracy would undoubtedly occur if an injection syringe of 3 to 5 microliter capacity were available that would assure injection of all the liquid into the flash vaporizer.

Table 2
Results of analysis of rumen fluid
diluted 50 percent with aqueous mixtures of VFA

Description of sample	mmole/100 ml fluid					
	acetic	propionic	n-butyric	iso-butyric	iso-valeric	n-valeric
	VFA in undiluted rumen fluid[1]					
Rumen fluid - Cow A	12.02	4.47	1.71	.03	.08	.42
Rumen fluid - Cow B	8.28	2.55	1.91	.02	.05	.36
	Expected VFA in diluted rumen fluid					
1 Cow A (no VFA added)	6.01	2.24	.86	.02	.04	.21
2 " " - level a[2]	7.35	2.77	1.21	.11	.10	.26
3 " " - " b	8.67	3.36	1.58	.18	.15	.30
4 " " - " c	10.01	3.95	1.96	.26	.20	.33
5 " " - " d	11.33	4.53	2.33	.33	.25	.22
6 Cow B (no VFA added)	4.14	1.28	.96	.01	.02	.18
7 " " - level a	5.24	1.86	1.34	.10	.10	.20
8 " " - " b	6.56	2.45	1.71	.17	.15	.24
9 " " - " c	7.90	3.04	2.09	.25	.20	.20
10 " " - " d	9.22	3.62	2.46	.32	.25	.16
	Observed VFA in diluted rumen fluid[1]					
1 Cow A (no VFA added)	6.03	2.18	.83	.03	.05	.22
2 " " - level a	7.60	2.85	1.26	.09	.09	.25
3 " " - " b	8.68	3.44	1.60	.16	.13	.28
4 " " - " c	10.19	4.08	2.02	.24	.20	.35
5 " " - " d	11.11	4.56	2.39	.34	.21	.16
6 Cow B (no VFA added)	3.92	1.27	.96	.02	.05	.16
7 " " - level a	5.14	1.75	1.26	.05	.05	.21
8 " " - " b	6.45	2.32	1.66	.13	.11	.24
9 " " - " c	7.96	2.94	2.02	.20	.14	.20
10 " " - " d	9.43	3.55	2.44	.28	.20	.17
Avg $\left(\frac{\text{observed}}{\text{expected}} \times 100\right)$ %	99.8	98.9	99.5			97.3
Standard deviation	2.6	3.3	3.3			10.4

[1]Avg of triplicate analyses

[2]

	mmoles VFA added/100 ml diluted rumen fluid					
	acetic	propionic	n-butyric	iso-butyric	iso-valeric	n-valeric
level a	1.32	.59	.38	.08	.05	.04
level b	2.64	1.18	...75	.15	.10	.08
level c	3.98	1.77	1.13	.23	.15	.11 (Cow A .04 (Cow B
level d	5.30	2.35	1.50	.30	.20	

Comments on column packings and instrumental conditions. The column packing, 10% 12-hydroxystearic acid on Fluoropak 80, used for the separations of VFA in aqueous solutions was very stable under the conditions of these experiments. At the beginning of the gas chromatographic work several column packings were prepared and tested in order to select the one most suitable for the separation of VFA in aqueous solutions. A liquid phase of Tween 80 + H_3PO_4 (Erwin et al., 1961) on Gas-Chrom P was found to be unsuitable. The water in the sample was eluted as a long tailing band. While the flame ionization detector was insensitive to water, the water had a cooling effect on the flame which interfered with the resolution of the fatty acids eluted after the water. Similar unsatisfactory results were obtained with the following column preparations: 10% diethylene glycol adipate + 2% H_3PO_4 on either Gas-Chrom P or Fluoropak 80, and 10 or 20% Tween 80 + 2% H_3PO_4 on Fluoropak 80. These results indicated that when aqueous solutions of VFA are chromatographed by gas-liquid chromatography the affinity of both the stationary liquid phase and the solid support for water are important considerations.

Differences apparently exist between the available solid supports of diatomaceous earths. This might explain why Gas-Chrom P was unsuitable when substituted for Chromosorb W in the column packing reported by Erwin et al. (1961). It is difficult to explain why the combinations of Tween 80 and Fluoropak 80 did not give satisfactory results. Perhaps the physical differences in the instrumentation also influence the choice of column packing.

The gas chromatograph (Kromo-Tog K-7) used in this work had certain limitations in comparison to the instruments discussed by several other workers. The internal diameter of the column was 3 mm as compared to approximately 6 mm in most instruments used in such work. Conversely the entire eluate from the column was passed to the detector whereas

several of the instruments with larger columns use a by-pass to regulate the flow to the detector. The manufacturer suggests that the sample size be no greater than 1 microliter whereas most researchers have injected 20 microliter samples. Other workers have used different column temperatures and carrier flows from those used in the present work.

Several attempts were made to increase the column temperature uniformly with time during an analysis in order to obtain sharper peaks for iso- and n-valeric acids. Even over relatively small temperature ranges (20^{o}), and at the conditions of analysis described previously, the base line shifted in a somewhat curvilinear manner. It was concluded that this technique would not be feasible at high sensitivities unless an instrument with compensating columns were available.

Summary

(1) The VFA in rumen fluid were estimated quantitatively by GLC on a column of 10% 12-hydroxystearic acid on Fluoropak 80.

(2) The precision of the method was comparable to other common analytical procedures. The sample injection technique was described as a potential source of error in the present method.

(3) The average recoveries of the VFA added to rumen fluids were 99.8, 98.9, 99.5 and 97.3% for acetic, propionic, n-butyric and n-valeric acids, respectively.

II. The Effect of Mineral Salts in the Drinking Water of Dairy Cattle on the Fat Content of Milk

Objects

1. To study the effects of a natural well water of high soda content, two prepared mineral salt solutions (simulated well waters) and demineralized water on the percent fat in milk and the proportions of VFA in the rumen.

2. To study the effect of substituting oat-barley silage for 75% of the mixed alfalfa-grass hay (dry matter basis) on the percent milk fat and proportions of VFA in the rumen of cows receiving test waters.

Experimental

Four lactating Jersey cows were used to study the following test waters:

I. A well water from a local dairy farm containing approximately 725 ppm "bicarbonates of soda, lime and magnesium."

II. City water with added salts: 375 ppm $NaHCO_3$; 375 ppm $KHCO_3$; 50 ppm Na_2SO_4; 50 ppm $MgSO_4$.

III. City water with added salts: 350 ppm Na_2CO_3; 350 ppm K_2CO_3; 50 ppm $CaSO_4$; 50 ppm $CaCO_3$; 50 ppm $MgCO_3$.

IV. Demineralized city water.

A study of the effects of feeding dry mixed hay versus a roughage mixture in which oat-barley silage replaced 75% (dry matter basis) of the hay allowance was superimposed on the water treatments. The four cows were allotted at random to the water treatments, and were on test over four periods of 28 days each as follows:

Period	1	2	3*	4*
Water	City	Test	Test	City
Ration	Hay + Conc.	Hay + Conc.	Hay + Silage + Conc.	Hay + Conc.

*Owing to an injury, the cow receiving test water III did not complete periods 3 and 4.

The cows were restrained in swivel stanchions, fed in individual mangers and milked two times per day. Dry roughage, or its dry matter equivalent weight of silage, was fed at the daily rate of 3.0 lb/100 lb liveweight, and concentrate (Table 3) at the rate of 0.5 lb/lb of milk in excess of 10 lb, in two equal portions at 6 AM and 4 PM. Water was provided ad libitum from buckets secured within the mangers. Cobalt-iodized salt blocks were available at all times. The composition of the hay, silage and concentrate is summarized in Table 3.

Table 3
Composition of ration ingredients[1] (sample basis)

	Alfalfa-grass hay	Oat-barley silage	Concentrate[2]
Dry matter, %	93.5	27.8	87.9
Gross energy, therms/lb	1.901	0.626	1.828
Crude protein, %	16.9	2.3	15.6
Ash, %	9.0	2.4	6.0
KNO_3, %		0.10	
pH		4.3	

[1]Gross energy of the ration ingredients was determined with a Parr Oxygen Bomb Calorimeter. Other chemical determinations were carried out by A.O.A.C. (1960) methods.

[2]Concentrate mixture: 52% ground oats; 20% ground barley; 15% linseed oil meal; 10% wheat bran; 1% cobalt-iodized salt; 0.1% limestone; 1.9% dicalcium phosphate.

Milk and milk fat production, and feed and water consumption were recorded daily. Milk fat was determined by the rapid detergent method (A.O.A.C., 1960). During the last week of each test period several rumen fluid samples were obtained by stomach tube and subsequently analyzed for molar proportions of the VFA by GLC as described on pages 27 to 31.

Results and Discussion

Animal performance. The data pertaining to the intake of the ration ingredients are summarized in Table 4. The average milk fat percentage and daily production are summarized graphically in Figure 3.

The test waters and the silage used in this trial apparently had no effect on the percentage fat in the milk. For the cow receiving water I, natural water containing 725 ppm of soda, greater differences existed in percent milk fat between the two control periods (1 and 4) than between the control periods and the test periods. For all cows the day to day variation in percent fat in milk was frequently large. This is reflected in the weekly values shown in Figure 3.

To a large degree the increases in the percent fat of the milk are associated with corresponding decreases in milk production. The cow receiving water II, high in bicarbonates of sodium and potassium, produced less milk, but milk with a higher percentage fat during periods 2 and 3, the periods when test water was part of the ration. The intake of gross energy was also decreased during these periods (Table 4). The cow receiving demineralized water during periods 2 and 3 declined consistently in milk production to less than 5 lb/day and was dried off at the end of the first week of period 4. The increase in fat percentage shown in Period 3 (Fig. 3) is likely an effect of the stage of lactation.

In all cases the intakes of crude protein were above the generally accepted requirements (National Research Council, 1956) of dairy cattle of similar weight and production. However, the calculated digestible energy intakes of the cows were below required levels in several instances as shown in Table 5.

Table 4
Ration consumption during the fourth week of each period

Water	Period	Average daily consumption					
		Water	Hay	Silage	Concentrate	Gross energy	Crude protein
		lb.	lb.	lb	lb	therms	lb
I	1	120.6	20.1	-	14.3	64.35	5.63
'well'	2	153.3	21.1	-	17.4	71.92	6.28
	3	120.3(153.1)*	6.5	45.4	18.0	73.68	4 95
	4	139.4	15.1	-	18.0	61.61	5.36
II	1	113.0	18.9	-	13.0	59.69	5.22
'bicarb-	2	93.4	16.1	-	10.0	48.89	4.28
onate'	3	93.1(117.7)*	6.0	34.1	9.0	49.21	3.19
	4	127.6	19.4	-	10.0	55.16	4.84
III	1	99.9	16.4	-	9.0	47.63	4.17
'carb-	2	112.9	19.9	-	9.0	54.28	4.76
onate'							
IV	1	86.4	21.6	-	4.0	48.37	4.27
'demin-	2	96.7	29.4	-	3.0	61.37	5.44
eralized'	3	54.1(95.8)*	7.5	57.7	1.0	52.21	2.76
	4	90.9	25.4	-	1.0	50.12	4.45

*Test water + silage water

Table 5
Estimated digestible energy consumption during the last 7 days of each period (therms/day)

Period	Test Water			
	I ('well')	II ('bicarbonate')	III ('carbonate')	IV (demineralized)
1 (control)	40.14(45.35)*	37.11(38.42)	29.03(33.50)	27.17(24.14)
2	45.48(44.04)	30.14(33.90)	32.46(30.98)	33.63(23.97)
3	46.25(42.49)	29.51(30.75)		27.34(19.74)
4 (control)	40.34(40.15)	33.43(32.80)		26.83(15.40)

*Bracketed values represent NRC Requirements (1956).

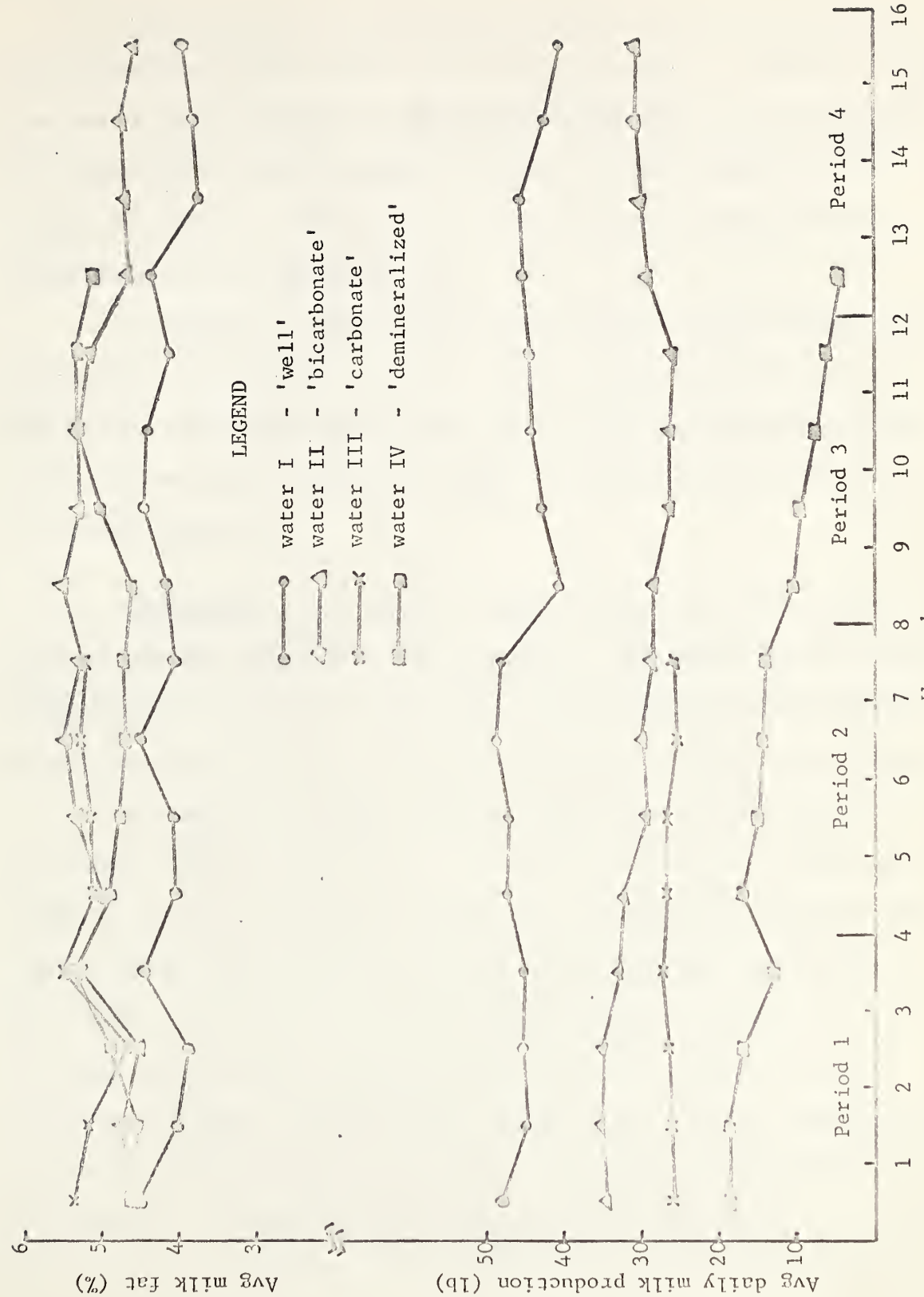

Figure 3. Average percent milk fat and milk production by periods.

The consumption of test waters decreased when silage replaced 75% of the dry roughage (dry matter basis) in period 3. However, the water content of the silage eaten was apparently equivalent to the decrease in the amount of test water consumed (Table 4). When a mixture of hay, silage and grain was offered to the cows, the dry hay and grain were preferentially and completely eaten.

In general, the test waters were consumed in amounts equal to or greater than the quantities of city water consumed in periods 1 and 4. The natural well water seemed particularly palatable and consumption in periods 2 and 3 was consistently higher than that of the city water in the control periods.

Ruminal VFA. The samples of rumen fluid obtained by stomach tube were of variable appearance and consistency. Presumably this was owing to difficulties in sampling the same area of the rumen and to the copious quantities of saliva which were secreted during the sampling operation.

Although the trends in the molar proportions of VFA (Table 6) are those often associated with a low percentage milk fat (Shaw et al., 1959) it is improbable that the small changes observed in this experiment would explain the low milk fat problem on the farm using the natural well water. The molar percent of acetate decreased from 68.0 to 65.0, and that of propionate increased from 19.6 to 21.9, when this water replaced city water. When silage was added to the ration containing the well water (period 3) the molar percent of propionic acid decreased to 18.9 and that of n-butyric acid increased to 13.2 from 10.8.

Table 6
Mean values for total VFA and molar proportions in the rumen

Water	Period	Total VFA (mmole/ 100 ml)	mole % acetic	pro-pionic	n-butyric	iso-butyric	iso-valeric	n-valeric
I	1	7.26	68.0	19.6	10.5	0.3	0.3	1.4
'well'	2	8.32	65.0	21.9	10.8	0.4	0.1	1.8
	3	7.42	64.3	18.9	13.2	0.7	1.3	1.6
	4	11.04	66.0	19.8	10.1	0.8	0.8	2.4
II	1	8.00	66.2	18.4	13.0	0.1	0.2	2.0
'bicarb-	2	9.96	63.4	20.1	14.6	0.2	0.1	1.7
onate'	3	7.87	65.2	19.1	14.0	0.3	0.3	1.3
	4	10.67	65.3	20.4	11.1	0.4	0.6	2.2
III	1	7.55	66.6	18.3	12.2	0.5	0.1	2.3
'carbonate'	2	9.36	66.7	19.4	10.8	0.5	0.3	2.2
IV	1	5.88	69.6	18.9	8.5	T*	0.7	2.4
'demin-	2	8.60	67.2	20.3	9.8	0.1	0.6	2.0
eralized'	3	5.89	68.3	18.2	11.0	T	1.2	1.4
	4	7.26	67.4	20.1	8.4	0.7	0.6	2.9

*T = < 0.1%

When the 'carbonate' water was added to the ration a slight trend toward an increase in the proportion of propionic acid and a decrease in n-butyric acid was observed while acetic acid was unchanged.

The results obtained with 'bicarbonate' water and demineralized water were inconsistent and do not suggest any changes in VFA relationships resulting from either the addition of test waters or the silage to the ration. In high grain milk fat-depressing rations, Emergy and Brown (1961) fed Na- or $KHCO_3$ at a much higher level (1 lb/day) than was consumed in the present experiment. The percentage milk fat returned to normal when the bicarbonates were fed. The molar proportions of the VFA, as determined with non-lactating cows, were unaltered. From these results they suggest that that the mechanism of Na- or $KHCO_3$ in preserving normal fat content of milk may not be associated with a decrease in the molar percent of propionic acid, but this suggestion is open to question because molar proportions

of VFA determinations were not made for the lactating cows. In the present experiment it seems that the increased fat level (Fig. 3) observed when the bicarbonate was used may have been more directly associated with the concurrent decrease in milk production than with changes in VFA.

Summary

(1) Within the limits of this experiment, the minerals either contained in or added to water had no measurable effect on the fat percentage of the milk of dairy cows.

(2) The addition of silage to the ration had no apparent effect on the percent fat in the milk.

(3) The molar percentages of VFA in the rumen fluid were generally variable and inconsistent. However, the natural well water was associated with a small decrease in the molar percent of acetate commensurate with an increase, of similar magnitude, in propionate. The molar percentage of propionate was increased slightly, and n-butyrate decreased slightly, when the high carbonate water replaced city water.

(4) The addition of silage to a ration containing the natural well water tended to depress the molar percentage of propionic acid and increase the molar level of n-butyric acid.

III. The Effects of Mineral Salts and Well Waters of High Mineral Salt Content on the Production of VFA *In Vitro*

Objects

1. To observe the effects of several common mineral salts, added singly or in combination, on VFA production in an *in vitro* rumen fermentation.

2. To compare the effects of well waters of high mineral salt content and distilled water on VFA production *in vitro*.

Experimental (General)

The *in vitro* rumen fermentation technique employed was similar to that described by Donefer, Crampton, and Lloyd (1960). Two fistulated dairy cows were used to obtain samples of ingesta from which inocula were prepared. The roughage (good quality mixed alfalfa-grass hay) and concentrate mixture (Appendix Table A) used as substrate in the *in vitro* studies were the same as those offered to the cows. The cow that provided the inoculum in Trial 1 was fed 4 lb concentrate mixture per day and hay plus water *ad libitum*. The inocula for all subsequent runs were obtained from a cow, fed 10 lb concentrate mixture per day and hay plus water *ad libitum*. Hay was fed long to the cows, whereas the hay-concentrate substrate used in the *in vitro* fermentation was ground to pass through a 20-mesh screen.

To prepare an inoculum, a quantity of rumen ingesta was obtained from a fistulated cow and the liquid expressed through six layers of cheese cloth by means of a mechanical fruit press. Approximately 6 lb of the pressed rumen pulp were resuspended in 1500 ml of phosphate buffer*, mixed thoroughly and pressed through six layers of cheese cloth with the

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*Phosphate buffer: 2.118 g Na_2HPO_4 + 0.872 g KH_2PO_4 + 0.200 g cysteine-hydrochloride per 2 liters of distilled water, heated to 40 C and saturated with carbon dioxide.

press. The phosphate buffer extract (PBE) was kept at a temperature of 40 C and used without further preparation as inoculum in the medium used to charge the *in vitro* rumen fermentation flasks.

At the completion of the 24-hour fermentation period, the fermentation solutions were acidified, centrifuged, and the concentrations of the VFA determined as described on pages 27-31. Samples of the nutrient medium-inoculum mixture were treated similarly in order to measure the pre-incubation levels of the VFA. The production of VFA was calculated from these data.

Four separate trials were conducted. The first two were concerned with the production of VFA from a substrate of 50% hay and 50% concentrate as influenced by individual mineral salts incorporated into the medium, singly or in combinations of two, at a level of 710 ppm each. Trials 3 and 4 were carried out to measure the effect of natural ground waters of high mineral salt content on the production of VFA from substrates composed of 1:1 and 9:1 hay-concentrate mixtures respectively. The data from treatments replicated in trials 1 and 2 were grouped together for a statistical analysis. Similarly the data of trials 3 and 4 were analyzed together.

Experimental, trial 1. In a 6 x 3 block-type experiment, the cations Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Zn^{++}, Co^{++}, and the anions Cl^{-}, CO_3^{-}, SO_4^{-} were studied in their possible salt combinations (Table 7) at a concentration of 710 ppm. Several other mineral salts and combinations of mineral salts were screened as shown in Table 8. Each salt was added at a final concentration of 710 ppm

One gram of substrate was weighed into each fermentation tube. Following addition of the appropriate mineral(s) to each tube, 5 ml distilled water were added to each tube to wet the substrate.

Table 7
Mineral salts studied in block experiment

NaCl	KCl	$CaCl_2$	$MgCl_2$	$ZnCl_2$	$CoCl_2$
Na_2CO_3	K_2CO_3	$CaCO_3$	$MgCO_3$	$ZnCO_3$	$CoCO_3$
Na_2SO_4	K_2SO_4	$CaSO_4$	$MgSO_4$	$ZnSO_4$	$CoSO_4$

Table 8
Miscellaneous mineral salts studied in trial 1

$Na_2CO_3 + Na_2SO_4$	$CoCO_3 + CoSO_4$
$K_2CO_3 + K_2SO_4$	$NaHCO_3$
$CaCO_3 + CaSO_4$	$KHCO_3$
$MgCO_3 + MgSO_4$	$NaNO_3$
$ZnCO_3 + ZnSO_4$	3 controls (no mineral salt)

The basal medium and inoculum (Table 9) were premixed in a 2-liter Erlenmeyer flask in quantities sufficient for the preparation of 40 fermentation tubes. The solutions were placed in a 2-liter flask in the order and proportions cited in Table 9, saturated with CO_2 at 40 C, placed on a magnetic stirrer and attached to an automatic pipette which dispensed 45 ml of the mixture to each fermentation tube, bringing the total volume up to 50 milliliters. After the addition of two drops of mineral oil to each tube to prevent foaming, the tubes were placed in a water bath at 40 C. During the 24-hour fermentation, CO_2 was bubbled through each tube independently at a rate of approximately 160 bubbles per minute. The approximate pH of each tube was determined with Alkacid test paper at the end of the 24-hour fermentation.

Table 9
Composition of in vitro basal medium-inoculum mixture, trial 1

Solution	Volume/tube (ml)
Mineral mixture*	10.0
Urea (12.6 g/100 ml)	0.5
Dextrose (10 g/100 ml)	0.5
Na_2CO_3 (200 g/liter)	1.5
Biotin (0.02 g/100 ml)	0.05
p-Aminobenzoic acid (0.05 g/100 ml)	0.05
Phosphate buffer extract (inoculum)	26.4
Iron and calcium (2.2 g $FeCl_3 \cdot 6H_2O$; 2.645 g $CaCl_2 \cdot 2H_2O$/500 ml)	0.5
n-Valeric acid (1 g/200 ml)	3.0
Casein hydrolysate-enzymatic (2 g/100 ml)	2.5

*Na_2HPO_4, 5.65 g; NaH_2PO_4, 6.27 g; KCl, 2.15 g; NaCl, 2.15 g; $MgSO_4 \cdot 7H_2O$, 0.582 g; Na_2SO_4, 0.75 g per liter.

Experimental, trial 2. The experimental techniques were those used in trial 1 except for several modifications to the nutrient medium. The concentration of dextrose stock solution was reduced to 5 g/100 ml, and the Na_2CO_3, n-valeric acid and casein hydrolysate were deleted from the medium. Thus, with the exception of dextrose, all fermentable substances were eliminated from the medium so that the substrate would be the main source of the VFA produced. The PBE inoculum was increased to 33.4 ml/tube to compensate for volume changes as a result of the above deletions.

Initially the Na_2CO_3 was withheld from the medium to allow each mineral to express its effect on the pH of the fermentation mixture. The pH of the mixture in each tube was measured at 3 and 8 hours after fermentation was begun. As the pH values ranged between 4.97 and 5.62 after 8 hours, 1 ml of the Na_2CO_3 solution (Table 9) was added to each tube at that

time to raise the pH of each mixture approximately 1 pH unit in order that fermentation would not cease because of an excessively acid medium. The pH values of the altered solutions were measured on the 9th and 24th hour of fermentation.

The mineral salts studied in this trial were the salts representing all combinations of cations, Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Zn^{++}, Co^{++}, and anions, Cl^{-}, CO_3^{--}, SO_4^{--}, (Table 7) and the salts listed in Table 10.

Table 10
Miscellaneous mineral salts studied in trial 2

$NaNO_3$	$Co(NO_3)_2$
KNO_3	$NaHCO_3$
$Ca(NO_3)_2$	$KHCO_3$
$Mg(NO_3)_2$	$NaHCO_3 + KHCO_3$
$Zn(NO_3)_2$	4 controls (no mineral salt)

Experimental, trial 3. Eight samples of the well water used as drinking water for lactating dairy cattle on farms in the Edmonton milk shed were collected and compared for their effects on VFA production *in vitro*. The chemical analyses for the eight samples are listed in Table 11. Water samples B to H inclusive were collected from farms classified as consistently producing milk containing less than 3.25 percent fat. Sample A was from a farm that had no milk fat problem.

A 9 x 2 x 2 factorial experiment was set up to compare the effects of natural waters or distilled water, two substrates (9:1 and 1:1 mixtures of hay and concentrate), and two levels of Na_2CO_3 (0 and 0.2 g/tube) in the fermentation medium on VFA production.

Table 11
Analyses of well water samples*

Determination	Water sample A	B	C	D	E	F	G	H
	(ppm)							
Total solids	1702	2296	1072	1340	880	1718	1038	1790
Ignition loss	330	266	268	84	214	130	52	222
Hardness	645	555	490	85	280	120	125	215
Sulphates	562	802	175	nil	153	nil	45	nil
Chlorides	15	40	33	430	nil	656	28	790
Alkalinity	625	775	645	555	485	535	870	350
Nitrites	Trace	Trace	Trace	0	0	0	Trace	0
Nitrates	Trace	10.7	21.4	0	0	0	0.42	0
Iron	1.5	0.2	0	0.2	3.+	1.5	0.2	0
Zinc	0	0	0	0.07	0.09	0	0	0.12

*Analyses were provided by the Provincial Analyst, University of Alberta, Edmonton.

The *in vitro* techniques, with several modifications, were similar to those in Trial 2. The mineral mixture (Table 9) was replaced with an equal volume of the test water in all fermentations except where the test water was distilled water, and nine PBE inocula were prepared using a different test water in each case instead of distilled water (see footnote page 45). The 5 ml water added initially to wet the substrate (see trial 1) was the specific test water allotted to that tube. In this way the aqueous portion of the *in vitro* fermentation solution was representative of the test water in question.

The tubes were charged with substrate and medium-inoculum mixture in the proportions cited in trial 2; however, owing to the fact that different test waters were used to prepare PBE, nine medium-inoculum mixtures were prepared in quantities sufficient for the preparation of five fermentation tubes each and dispensed separately to the respective tubes. The two levels of Na_2CO_3, in 1 ml water, were added to the respective tubes prior to placing the tubes in a water bath. The pH of

the fermentation mixture in each tube was observed at 3, 8 and 24 hours after fermentation was begun, however pH adjustments were not made after 8 hours.

Experimental, trial 4. Trial 4 was a repeat of trial 3 with three modifications: (1) Two hundred fifty ml of the test water allotted to each tube were evaporated to approximately 50 ml in a 400 ml beaker, transferred to the fermentation tube and evaporated to a volume of 5 milliliters. The substrates were added to the concentrated solutions in the tubes. (2) The phosphate buffer extract-nutrient medium mixture was prepared and dispensed as in trial 2, and (3) The mineral mixture (Table 9) was omitted from the fermentation medium dispensed to all tubes. The net effect of these modifications was to concentrate the total solids found in the original water, or present in the fermentation tubes of trial 3, five-fold.

Results and Discussion

Trials 1 and 2. The average values for VFA produced in vitro as influenced by the cations and anions listed in Table 7 are shown in Table 12. The mean squares of the analyses of variance of the data are shown in Appendix Table B.

Zn or Co salts altered the production of acetic, propionic, n-butyric and n-valeric acids compared to Na , K , Ca or Mg salts (Table 12). The average molar percentages of the VFA in fermentations containing the ions Na^{+}, K^{+}, $Ca^{++}$and Mg^{++} versus Zn^{++} and Co^{++} were, respectively, acetic 58.7 vs. 47.7, propionic 25.1 vs. 39.8, n-butyric 13.4 vs. 7.7, and n-valeric 1.7 vs. 4.0. Zn^{++} was associated with a lower production of total VFA, acetic acid, propionic acid, and n-valeric acid than Co^{++}. However, the molar proportions of the individual acids produced in the presence of Zn^{++} or Co^{++} were very similar, Co^{++} being

associated with a slightly higher proportion of n-butyric acid and a slightly lower proportion of propionic acid compared to Zn^{++}.

VFA production (mmole/100 ml) in the presence of Na^{+} tended to be greater than in the presence of the other ions tested. The Na^{+} effect was the most pronounced ($P < .05$) on acetic acid and on total VFA production.

Table 12
The effects of cations and anions on VFA production in vitro, trials 1 and 2

	VFA						
Ion	Total VFA	acetate	propio-nate	n-butyrate	iso-butyrate	iso-valerate	n-valerate
			(mmole/100 ml)				
Cations							
Na^{+}	10.28[d]	6.07[d]	2.56[a]	1.37[c]	.03	.07	.18[a]
K^{+}	9.66[bc]	5.67[c]	2.47[a]	1.26[c]	.03	.08	.14[a]
Ca^{++}	9.84[cd]	5.76[cd]	2.43[a]	1.36[c]	.03	.08	.18[a]
Mg^{++}	9.60[bc]	5.61[c]	2.42[a]	1.29[c]	.03	.08	.16[a]
Zn^{++}	8.03[a]	3.84[a]	3.24[b]	.57[a]	.02	.03	.32[b]
Co^{++}	9.14[b]	4.35[b]	3.58[c]	.76[b]	.02	.06	.36[b]
			(mole %)				
Na^{+}		59.0	24 9	13.3	.3	.7	1.8
K^{+}		58.7	25.6	13.1	.3	.8	1.5
Ca^{++}		58.6	24.7	13.8	.3	.8	1.8
Mg^{++}		58.5	25.2	13 5	.3	.8	1.7
Zn^{++}		47.8	40.4	7.1	.3	.4	4.0
Co^{++}		47.6	39.2	8.3	.2	.7	4.0
			(mmole/100 ml)				
Anions							
Cl^{-}	9.48	5.14	2.92[a]	1.09	.03	.07	.23
CO_3^{--}	9.40	5.34	2.62[b]	1.14	.02	.07	.22
$SO_4^{=}$	9.38	5.17	2.82[a]	1.08	.03	.06	.23
			(mole %)				
Cl^{-}		54.2	30.8	11.5	.3	.8	2.4
CO_3^{--}		56.8	27.9	12.1	.2	.7	2.3
$SO_4^{=}$		55.1	30.1	11.5	.3	.6	2.4

[abcd]Values with the same superscript, within a column subgroup, are not significantly different ($P > .05$).

Significantly less ($P < .05$) propionic acid was produced in the presence of CO_3^{--} than in the presence of Cl^- or SO_4^{--} (Table 12). The effect of CO_3^{--} on propionic acid production was more pronounced in the presence of Zn^{++} and Co^{++} than of Na^+, K^+, Ca^{++} and Mg^{++}. The mean amounts of propionate produced in the presence of Zn^{++} and Co^{++} were 3.72, 2.96 and 3.56 mmole per 100 ml for their Cl^-, CO_3 and SO_4 salts respectively (Appendix Tables C and D). In comparison, mean amounts of 2.52, 2.44 and 2.44 mmole propionate per 100 ml were produced in the presence of Cl^-, CO_3^{--} and SO_4^{--}, respectively, when present as their Na^+, K^+, Ca^{++} and Mg^{++} salts. Several second order interactions were found to be significant ($P < .05$). However, it would appear from these data that most ions commonly found in the ration do not interact in their effects, if any, on VFA production.

The amounts of total VFA, acetate, propionate, n-butyrate and iso-butyrate produced were significantly less ($P < .05$) in trial 2 than in trial 1 (Appendix Table B). The reduced production of VFA in trial 2 may be attributable to the reduction in the amount of dextrose and the deletion of casein hydrolysate and n-valeric acid from the nutrient medium. The average production, as calculated from the data in Appendix Tables C and D, in mmoles per 100 ml per hour of acetate, propionate, n-butyrate and n-valerate in trials 1 and 2 were 0.25 and 0.19, 0.12 and 0.11, 0.05 and 0.04 and 0.01 and 0.01, respectively. It is interesting to note that propionate and valerate were not influenced to as great a degree as were acetate and n-butyrate. While the rates of VFA production were sufficient to produce total concentrations in 24 hours comparable to those often observed *in vivo*, the rates of production of acetate determined in this work were much lower than the values of 1.79 and 1.85 mmoles per 100 ml per hour obtained *in vivo* by Sheppard *et al.* (1959) and Gray *et al.* (1960) respectively, with sheep.

The VFA data from the control fermentation (no mineral salts added) and those supplemented with two mineral salts, bicarbonates or nitrates, are summarized in Table 13. The differences in VFA production between trial 1 and trial 2 discussed earlier are evident in the control fermentations; a lower production of total VFA, acetate and n-butyrate (total VFA x mole %, Table 13) in trial 2 with little difference in the production of propionate or valerate.

A mixture of the carbonate and sulfate salts of each of the cations Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Zn^{++} and Co^{++} had the same effect on VFA production as a single addition of each salt. The total VFA production at a mineral salt concentration of 1420 ppm was comparable to that at a salt concentration of 710 ppm (Tables 12 and 13).

The production of VFA in <u>in vitro</u> rumen fermentations containing Na- and/or $KHCO_3$ was not different from that in the unsupplemented fermentations (Table 13). On the other hand nitrates were associated with a depressed VFA production and a trend to lower percentages of acetic and n-butyric acids commensurate with higher percentages of propionic and n-valeric acids (Table 13). Jamieson (1959) reported that the oral administration of nitrate to sheep on pasture induced similar changes in the relative proportions of acetic and propionic with little change in the other acids.

The pH of the 24-hour fermentation mixtures in trial 1 was neutral as determined with "Alkacid" test paper. The average pH values of the fermentation solutions of trial 2 are summarized in Table 14. In general salts did not influence the pH of the fermentation mixtures to any extent. The fermentation mixtures containing Zn^{++} were the exception with an average 24-hour pH of 6.09 versus 6.32 for controls.

Table 13
Total VFA production and molar proportions, miscellaneous trials 1 and 2

Description	Trial	Mineral salt(s)	Total VFA (mmole/100 ml)	mole % acetic	propionic	n-butyric	iso-butyric	iso-valeric	n-valeric
Controls	1	None (avg 3 tubes)	10.89	59.9	23.4	14.2	.4	.6	1.5
	2	None (avg 4 tubes)	9.05	56.3	27.5	13.4	.2	.7	1.9
Combinations*	1	$Na_2CO_3 + Na_2SO_4$	9.82	60.6	21.1	15.9	.3	.3	1.8
	1	$K_2CO_3 + K_2SO_4$	10.27	59.7	21.8	14.8	.5	.6	2.6
	1	$CaCO_3 + CaSO_4$	11.03	58.8	24.3	13.7	.5	1.0	1.7
	1	$MgCO_3 + MgSO_4$	11.31	58.6	23.7	15.0	.5	.6	1.6
	1	$ZnCO_3 + ZnSO_4$	8.02	41.5	48.0	6.5	.3	1.2	2.5
	1	$CoCO_3 + CoSO_4$	9.44	43.1	46.9	4.9	.2	.3	4.6
Bicarbonates	1	$NaHCO_3$	10.81	59.9	23.3	13.7	.5	.8	1.8
	2	"	9.41	58.0	28.1	11.7	0	.6	1.6
	1	$KHCO_3$	9.03	58.1	25.4	12.4	.2	.6	3.3
	2	"	10.15	56.9	27.9	12.4	.2	.7	1.9
	2	$NaHCO_3 + KHCO_3$	8.51	59.5	26.4	11.1	.2	.9	1.9
Nitrates	1	$NaNO_3$	9.46	53.8	27.6	14.3	.2	.3	3.8
	2	"	6.70	56.4	28.4	9.7	0	.3	5.2
	2	KNO_3	8.07	56.0	29.5	10.5	0	.1	3.9
	2	$Ca(NO_3)_2$	7.73	53.7	30.1	11.5	0	.4	4.3
	2	$Mg(NO_3)_2$	7.77	59.7	27.2	9.8	0	.5	2.8
	2	$Zn(NO_3)_2$	5.62	47.7	39.9	8.7	0	0	3.7
	2	$Co(NO_3)_2$	6.51	47.8	37.6	8.9	.1	.5	5.1

*The concentration of each mineral salt was 710 ppm

Table 14
The pH of the *in vitro* fermentation solutions, trial 2

Criterion		avg pH 3-hour	8-hour		9-hour	24-hour
Cations	Na^+	5.47	5.31	*	6.28	6.40
	K^+	5.66	5.31		6.24	6.46
	Ca^{++}	5.46	5.20		6.14	6.31
	Mg^{++}	5.65	5.34		6.26	6.39
	Zn^{++}	5.35	5.09		6.22	6.09
	Co^{++}	5.57	5.49		6.40	6.33
Anions	Cl^-	5.51	5.27		6.29	6.30
	CO_3^{--}	5.60	5.29		6.24	6.42
	SO_4^{--}	5.52	5.19		6.18	6.26
	NO_3^-	5.49	5.41		6.33	6.33
Control	(avg 4 tubes)	5.56	5.21		6.30	6.32
Bicarbonates	$NaHCO_3$	5.63	5.13		6.31	6.21
	$KHCO_3$	5.64	5.14		6.38	6.37
	$NaHCO_3 + KHCO_3$	5.64	5.33		6.42	6.37

*0.2 g Na_2CO_3/1 ml added to each fermentation mixture after 8 hours to raise pH approximately 1 pH unit.

Trials 3 and 4. The effects of the natural well waters, substrates, Na_2CO_3 and trials on the production of VFA *in vitro* and on the molar proportions of the VFA are summarized in Tables 15 and 16, respectively. The mean squares of the analyses of variance are listed in Appendix Table E.

Distilled water and the natural well waters were not significantly different ($P > .05$) in their effects on the production of total VFA, acetate, propionate, iso-valerate or n-valerate. Well waters F and G were associated with a significantly lower ($P < .05$) production of n-butyrate than distilled water and five of the other well waters, the remaining well water, D, was associated with a higher production of n-butyrate but not significantly higher ($P > .05$). An analysis of variance of the data from trial 4, where the solids in the waters were concentrated five-fold, did not indicate any significant differences in the production of VFA between the nine waters tested (Appendix Table F).

Table 15
The effects of natural well waters, substrates, Na_2CO_3 and trials on VFA production and pH *in vitro*, trials 3 and 4

Comparison	No. determinations	Total VFA (mmoles/100 ml)	acetic	propionic	n-butyric	iso-butyric	iso-valeric	n-valeric	24-hour pH
Water:									
Distilled	8	8.38	5.12	1.90	1.05[a]	.04[a]	.05	.22	5.62[e]
A	8	8.29	5.16	1.96	.94[ab]	.02[b]	.04	.18	5.89[c]
B	8	8.30	5.20	1.87	.93[ab]	.04[a]	.05	.22	6.02[ab]
C	8	7.87	4.78	1.91	.92[ab]	.02[b]	.03	.21	6.00[b]
D	8	7.94	4.94	1.90	.83[bc]	.02[b]	.06	.19	5.99[b]
E	8	8.53	5.30	2.03	.94[ab]	.03[ab]	.04	.19	5.79[d]
F	8	7.18	4.56	1.69	.73[c]	.02[b]	.03	.16	5.93[bc]
G	8	7.66	4.93	1.71	.77[c]	.04[a]	.05	.17	6.11[a]
H	8	8.05	4.91	1.84	.98[a]	.04[a]	.08	.20	5.77[d]
Substrate:									
1 hay:1 conc	36	8.12	4.89	1.97	.99[a]	.034[a]	.05	.19	5.85[a]
9 hay:1 conc	36	7.93	5.08	1.77	.81[b]	.028[b]	.04	.19	5.95[b]
Na_2CO_3:									
0	36	7.85	4.76[a]	1.86	.97[a]	.03	.05	.19	5.58[a]
0.2g	36	8.20	5.22[b]	1.88	.83[b]	.03	.05	.20	6.22[b]
Trial:									
3	36	8.28	5.24[a]	1.87	.85[a]	.04[a]	.07[a]	.21[a]	5.55[a]
4	36	7.76	4.73[b]	1.86	.95[b]	.02[b]	.03[b]	.17[b]	6.25[b]

[abcde] Values with the same superscript within a comparison subgroup are not significantly different ($P > .05$)

Table 16
The effects of natural well waters, substrates, Na_2CO_3 and trials on molar proportions of VFA *in vitro*, trials 3 and 4

	mole percent					
	acetic	propionic	n-butyric	iso-butyric	iso-valeric	n-valeric
Water:						
Distilled	61.1	22.7	12.5	.5	.6	2.6
A	62.2	23.6	11.3	.2	.5	2.2
B	62.6	22.5	11.2	.5	.6	2.6
C	60.7	24.3	11.7	.2	.4	2.7
D	62.2	23.9	10.5	.3	.7	2.4
E	62.1	23.8	11.0	.4	.5	2.2
F	63 5	23.5	10.2	.2	.4	2.2
G	64.4	22.3	10.0	.5	.6	2.2
H	61.0	22.8	12.2	.5	1.0	2.5
Substrate:						
1 hay:1 conc	60.2	24.3	12.2	.4	.6	2.3
9 hay:1 conc	64.1	22.4	10.2	.4	.5	2.4
Na_2CO_3:						
0	60.6	23.7	12.3	.4	.6	2 4
0.2 g	63.6	22.9	10.1	.4	.6	2.4
Trial:						
3	63.3	22.6	10.3	.5	.8	2.5
4	61.0	24.0	12.2	.2	.4	2.2

There was a tendency for the natural well waters to be associated with a small decrease in the molar proportion of n-butyric acid compared to distilled water (Table 16). The waters associated with the lowest production of n-butyrate were also those with the lowest molar proportions of n-butyrate. In general expressing results in terms of molar percentages tends to mask the VFA production values. A low production of one acid causes the proportions of the other acids to change, thus masking the real effect, if any, of a treatment on the production of the other individual VFA. Those waters associated with the lowest total VFA production were also associated with the greatest differences in the molar proportions of the VFA produced. In several instances, e.g. waters F and G, the molar proportions of the VFA were not those expected under conditions of low milk fat (Table 16).

The VFA production data from the *in vitro* fermentations indicate that the natural well waters are not likely to alter the production of VFA to any marked degree. An examination of the chemical analyses of the waters (Table 11) did not reveal any consistent relationships between their chemical entities and the production of VFA.

The total VFA produced from the two substrates were not significantly different ($P > .05$) nor were there any significant interactions between substrates and test waters. The production of acetate was lower and that of propionate and n-butyrate higher on the 1:1 hay-concentrate than the 9:1 hay-concentrate substrate (Table 15). However, of these only the n-butyrate difference was significant ($P < .05$).

When Na_2CO_3 was added to the fermentation solutions the total VFA production was greater than when Na_2CO_3 was not added. The Na_2CO_3 was associated with a significantly higher ($P < .05$) acetate and a significantly lower ($P < .05$) n-butyrate production. The production of propionate and n-valerate was unchanged by the Na_2CO_3.

Significantly less acetate, iso-butyrate, iso-valerate and n-valerate and significantly more n-butyrate were produced in trial 4 than in trial 3. The production of propionate was unchanged from trial 3 to trial 4. Na_2CO_3 appeared to have a different effect in trial 3 than in trial 4. In trial 3 Na_2CO_3 had a stimulating effect, whereas in trial 4 it had a depressing effect on VFA production (Table 17). The main difference in conditions between trials 3 and 4 was the five-fold concentration of the solids in the well waters in trial 4 versus trial 3. The average 24-hour pH without Na_2CO_3 was 5.92 in trial 4 versus 5.86 with Na_2CO_3 in trial 3. These similar pH values were associated with the highest production of the VFA (Table 17).

The pH values of the test waters and the fermentation solutions are summarized in Table 18. The natural well waters varied in pH from

7.07 to 8.21; the mean pH values of the fermentation solutions were not necessarily related to the original pH values of the respective waters. The range in 24-hour average pH values for the fermentations with the natural waters was 5.77 to 6.11 while the average pH of the distilled water fermentations was 5.62.

Table 17
The effect of Na_2CO_3 in trials 3 and 4

Trial	Na_2CO_3	24-hour pH	avg VFA production (mmole/100 ml) Total VFA	acetic	propionic	n-butyric
3	0	5.24	7.02	4.30	1.60	0.84
	0.2 g/tube	5.86	9.54	6.18	2.15	0.86
4	0	5.92	8.68	5.21	2.12	1.09
	0.2 g/tube	6.59	6.85	4.26	1.60	0.80

Table 18
The pH of distilled water, natural well waters and *in vitro* rumen fermentation mixtures containing the test waters

	pH of water	avg pH of fermentation mixtures 3-hour	8-hour	24-hour
Water:				
Distilled	6.43	5.91	5.70	5.62
A	7.07	6.14	6.00	5.89
B	7.14	6.27	6.08	6.02
C	7.35	6.29	6.10	6.00
D	7.87	6.25	6.06	5.99
E	7.21	6.13	5.92	5.79
F	7.70	6.21	6.01	5.93
G	8.21	6.33	6.22	6.11
H	7.39	6.01	5.85	5.77

Summary

(1) In rumen fermentations *in vitro*, Zn^{++} and Co^{++} were associated with a lesser production of acetate and n-butyrate commensurate with a greater production of propionate and n-valerate as compared to Na^{+}, K^{+}, Ca^{++} and Mg^{++}. The molar proportions of the VFA were of the same order as the production of the VFA. Total VFA production was significantly less ($P < .05$) in the presence of Zn^{++} as compared to the other cations. The VFA production associated with Co^{++} was less than that with Ca^{++} or Na^{++}, and similarly that with K^{+} and Mg^{++} was less than that with Na^{+}.

(2) Significantly less ($P < .05$) propionic acid was produced in the presence of CO_3^{--} than in the presence of Cl^{-} or SO_4^{--}. No other significant effects on VFA production were associated with the anions Cl^{-}, CO_3^{--} and SO_4^{--}.

(3) A cation added to a fermentation tube as a mixture of its carbonate and sulfate salts produced effects of the same magnitude as when added at one-half the ionic strength as a single mineral salt.

(4) The bicarbonates of Na and/or K had no apparent effect on VFA production *in vitro*.

(5) Nitrate ions appeared to decrease VFA production *in vitro*. The relative proportions of acetic and n-butyric acids were lowered whereas the relative proportions of propionic and n-valeric acids were increased in the presence of NO_3^{-}.

(6) The mineral salts tested in the *in vitro* fermentations had little effect on the pH of the fermentation solutions.

(7) The nine waters (8 well waters + distilled water) tested were not significantly different ($P > .05$) in their effect on the production of the VFA except for n-butyric and iso-butyric acids. The production and relative proportion of n-butyric acid tended to be higher with distilled water than with the various well waters, whereas a specific trend was not obvious with iso-butyric acid.

IV. The Effects of Mineral Salts on VFA and the Intra-Rumen Environment *In Vivo*

Objects

1. To study the effects of several mineral salts, in high concentrations in the drinking water of cattle, on the total amounts and proportions of VFA in the rumen at various time intervals after feeding.

2. To estimate the relative rates of VFA production under the treatment regimens.

3. To measure the effects of mineral salts on rumen pH, dry matter and fill at various times after feeding.

4. To measure feed and water consumption as affected by the addition of mineral salts to the drinking water.

Experimental

Two fistulated Jersey cows, A in early lactation and B dry (6 months pregnant), were used. Each treatment water was fed for 7 days, a 4½-day preliminary period followed by a 2½-day controlled feeding test period. The treatments outlined in Table 19 were administered sequentially, as allotted to each cow, with a new one beginning immediately upon completion of the preceding one. In addition to the test waters Cow A received 12 lb and Cow B 3 lb of the concentrate mixture (Appendix Table A) per day throughout these experiments. Long alfalfa-grass hay and the test waters, given in buckets secured within the feeding manger, were provided *ad libitum* during the preliminary periods and for 2 hours twice per day during the controlled feeding periods. One-half the ration was provided to the cows at each feeding, 6 AM and 4 PM during the preliminary period and 5 AM and 5 PM during the controlled feeding period.

Table 19
Description and allotment of water treatments to cows

Cow	Treatment water	Treatment description: Mineral salt(s)	Treatment description: Level in water (ppm)	Total salt (grains/gallon)
A	Control	None		
	Zn salts	$ZnCl_2$	237	
		$ZnCO_3$	237	50
		$ZnSO_4$	237	
	$NaHCO_3$	$NaHCO_3$	1420	100
	Ca salts	$CaCl_2$	237	
		$CaCO_3$	237	50
		$CaSO_4$	237	
	Na- + K phosphates	Na_2HPO_4	355	
		NaH_2PO_4	355	100
		K_2HPO_4	355	
		KH_2PO_4	355	
B	Control	None		
	Na salts	NaCl	473	
		Na_2CO_3	473	100
		Na_2SO_4	473	
	Mg salts	$MgCl_2$	237	
		$MgCO_3$	237	50
		$MgSO_4$	237	
	Co salts	$CoCl_2$	237	
		$CoCO_3$	237	50
		$CoSO_4$	237	

During the final 2½ days on each treatment one-half of the grain allowance and hay plus water ad libitum were available to the cows for 2 hours at each feeding interval. At the end of the 2-hour feeding period the hay and water not consumed were weighed back, and the consumption recorded; the grain allowance was always completely eaten. The cows remained without feed or water for 10 hours until the next feeding period. The strict feeding schedule served to accustom the cows to a routine in preparation for the critical final 12 hours of each treatment regimen,

during which time estimates were made of the relative rates of production of VFA, in conjunction with a study of other rumen factors, at six intervals.

Samples of rumen ingesta were removed and incubated *in vitro* for 1 hour under CO_2. Fluid from the rumen ingesta in the *in vitro* jars was subsampled for VFA analysis immediately (0-hour *in vitro*) and at 30 and 60 minutes. The 0-hour *in vitro* samples represented the concentrations of VFA in the rumen at the time of sampling. The increase in the concentration of a fatty acid *in vitro* from ½-hour to 1-hour was used to estimate the relative rate of production of that fatty acid.

The *in vitro* apparatus was similar to that described in part III, page 45. The 90 ml fermentation tubes were replaced with 350 ml wide-mouthed cylindrical jars fitted with No. 12 two-hole rubber stoppers. One hole served as an inlet for the carbon dioxide tube and the second as a gas outlet and sampling port. Sampling of the rumen fluid was accomplished by mixing the contents of the jar and quickly pouring 10 ml rumen fluid out through the sampling port in the rubber stopper.

Two 200 to 250 g representative samples of ingesta were obtained from the medial area of the ventral sac of the rumen at six different times during the final 12-hour period on each treatment regimen; 0 hour, immediately after the orts were removed (2-hour) and at 4, 6, 8 and 10 hours after the 0-hour sample was obtained. The samples were mixed, sampled and incubated as described above. Equal quantities of the duplicate rumen liquid subsamples were pooled for the VFA analysis. A third representative sample of ingesta (150-200 g) was withdrawn concurrently from the same area of the rumen in a beaker for pH and dry matter determinations. The water intake during the feeding interval at the beginning of the collection period was limited to one-quarter of the total consumed during the four previous 12-hour intervals, so that great fluctuations in consumption did not occur.

The weight of the rumen contents was determined at three separate times in the 12-hour period following the collection period described above. The rumen was emptied and the contents weighed, mixed, sampled for a dry matter determination, and returned to the rumen. This was completed immediately before feeding (0-hour) and at 3 and 6 hours after feed was first offered. Any feed and water not consumed after 2 hours was removed from the manger and not returned until after the 6-hour rumen fill measurement had been completed.

Samples of rumen fluid were prepared and analyzed for VFA by the procedures described on pages 27-31. Dry matter of the rumen content was determined by a standard A.O.A.C. method. The total volume of water in the rumen was plotted graphically so that its volume at intermediate times could be estimated. The pH of rumen ingesta was determined within 1 minute after the sample was obtained using a Beckman Model G pH meter fitted with a probe electrode assembly.

The milk production and percent fat in the milk of cow A were recorded during the controlled feeding period of each treatment regimen. Milk fat was determined by the rapid detergent method (A.O.A.C., 1960).

Results and Discussion

The mean effects of several mineral salts in the drinking water on ration consumption, water and dry matter in the rumen and rumen pH are summarized in Table 20. The mean concentrations, amounts and energy equivalents of the VFA are presented in Table 21. Mean squares of the analyses of variance of the data from Tables 20 and 21 are summarized in Appendix Tables G and H, respectively.

The presence of Zn or Co salts in the rations caused a progressive anorexia in both cows which was particularly apparent with Co. In view of the apparent toxicity of the Zn and Co salts at the levels used in this

experiment (710 ppm), the results obtained with these salts are confounded with a reduced intake of the ration (Table 20) and the effect of this on metabolism within the rumen.

It is interesting to note a converse situation existing between the two cows in water consumption (Table 20). Cow A did not consume more water when the minerals were added to the water than during the control period. Cow B increased water consumption by 87 and 70% when the Na and Mg salts, respectively, were added to the drinking water.

As shown in Table 20 the volume of water found in the rumen was consistently higher when mineral salts were dissolved in the drinking water. With the exception of Zn salts with Cow A, the addition of mineral salts to the drinking water of either cow resulted in a significantly decreased ($P < .05$) percent dry matter of rumen ingesta as compared to the control regimens. Zinc salts were associated with a slight, but non-significant ($P > .05$), increase in percent dry matter in the rumen as compared to the control.

When $NaHCO_3$, Ca salts or Na- + K phosphates were fed to Cow A (lactating) the concentrations of VFA in the rumen fluid were higher than during the control period (Table 21). On the other hand, the effects of different mineral salts on the levels of VFA in the rumen fluid of Cow B (dry) were less marked. The VFA concentrations on the mineral regimens did not differ significantly ($P > .05$) from the control, except for acetic and propionic acids in the presence of Co salts (Table 21).

Table 20
The mean effects of mineral salts in the drinking water on ration consumption and intra-rumen environment

	Cow A					Cow B			
	Control	Zn salts	$NaHCO_3$	Ca salts	Na- + K phosphates	Control	Na salts	Mg salts	Co salts
	Ration consumption (lb/day)								
Hay	16.7	13.2	17.5	18.3	18.4	16.8	19.0	18.9	9.4
Concentrate	12.0	12.0	12.0	12.0	12.0	3.0	3.0	3.0	3.0
Water	104	74	109	103	102	54	101	92	39
	Volume of water in the reticulo-rumen (liters)								
	33.66^{a}	39.85^{d}	35.84^{ab}	38.47^{cd}	36.90^{bc}	26.65^{x}	30.90^{xy}	34.38^{y}	32.80^{y}
	pH of rumen ingesta								
	6.01^{bc}	6.17^{c}	5.56^{a}	5.74^{ab}	5.72^{a}	6.73^{z}	6.51^{x}	6.60^{xy}	6.69^{yz}
	Dry matter of rumen ingesta (%)								
	16.5^{c}	17.1^{c}	15.0^{b}	14.3^{a}	15.2^{b}	15.1^{y}	12.1^{x}	12.1^{x}	12.0^{x}

^{abcd}Within Cow A, means on the same line with the same superscript letter do not differ significantly $(P > .05)$.
^{xyz}Within Cow B, " " " " " " " " " " " " " " $(P > .05)$.

When the total quantities of the VFA in the rumen were compared, rather than the concentrations per unit volume of fluid, it was found that the effects of minerals on the VFA in the rumen were accentuated. The amounts of total VFA, acetate, propionate and n-butyrate in the rumen were, for the most part, significantly greater ($P < .05$) when minerals were included in the ration (Table 21). Significant differences were not obtained between controls and treatments for n-butyrate in the presence of Zn salts (Cow A) and propionate and n-butyrate in the presence of Na salts, and total acid, acetate and propionate in the presence of Co salts (Cow B). The toxicity of Zn and Co salts cited previously may explain their apparent divergence from the general effect of minerals on the quantities of VFA in the rumen.

In order to obtain a value for the total energy of the VFA, present in varying quantities and proportions in the rumen, the gross caloric equivalents of the total VFA were calculated from the individual heats of combustion and the amounts of the VFA present (Table 21). The mean gross energy values were of the same relative order as were the moles of total VFA in the rumen.

There were significantly more ($P < .05$) moles of VFA in the rumen on the $NaHCO_3$ than on the Na- and K phosphates regimen, but in terms of the calculated energy values of the VFA the difference was not significant (Table 21). The greater proportion of n-butyrate in the VFA associated with the Na- + K phosphates than with the $NaHCO_3$ regimen would contribute to the change in significance. The mean heats of combustion of total VFA for cow A, as calculated from Table 21, were 296.0, 295.2, 291.0, 291.5 and 295.1 kcal/mole for the control, Zn, $NaHCO_3$, Ca and Na- + K phosphates regimens, respectively. For cow B values of 288.5, 286.4, 286.0 and 293.3 kcal/mole were calculated for control, Na, Mg and Co regimens, respectively.

Table 21
The mean effects of mineral salts in the drinking water
on concentrations, total quantities and caloric equivalents of VFA in the rumen

	Cow A					Cow B			
	Control	Zn salts	$NaHCO_3$	Ca salts	Na- + K phosphates	Control	Na salts	Mg salts	Co salts
Concentrations (mmoles/100 ml rumen fluid)									
Total VFA	12.96^{a}	12.67^{a}	18.83^{c}	15.74^{b}	16.07^{b}	9.87	10.64	10.51	8.98
acetic	8.38^{a}	8.28^{a}	12.42^{c}	10.36^{b}	10.49^{b}	6.68^{xy}	7.30^{y}	7.18^{y}	5.97^{x}
propionic	2.36^{a}	2.36^{a}	3.49^{c}	2.95^{b}	2.77^{ab}	1.66^{y}	1.75^{y}	1.80^{y}	1.48^{x}
n-butyric	1.82^{b}	1.47^{a}	2.42^{c}	1.97^{b}	2.38^{c}	1.26	1.24	1.23	1.26
iso-butyric	$.06^{b}$	$.07^{b}$	$.05^{ab}$	$.05^{ab}$	$.03^{a}$	.05	.06	.05	.05
iso-valeric	$.10^{a}$	$.15^{b}$	$.13^{b}$	$.10^{a}$	$.08^{a}$	.08	.09	.08	.08
n-valeric	.24	.34	.32	.31	.32	.14	.20	.17	.14
Quantities (moles)									
Total VFA	4.232^{a}	4.979^{b}	6.480^{d}	5.942^{cd}	5.785^{c}	2.600^{x}	3.229^{yz}	3.592^{z}	2.896^{xy}
acetic	2.734^{a}	3.254^{b}	4.274^{d}	3.909^{cd}	3.779^{c}	1.758^{x}	2.212^{yz}	2.452^{z}	1.921^{xy}
propionic	$.783^{a}$	$.928^{b}$	1.210^{d}	1.124^{cd}	1.006^{bc}	$.438^{x}$	$.540^{xy}$	$.621^{y}$	$.483^{x}$
n-butyric	$.586^{a}$	$.579^{a}$	$.824^{bc}$	$.736^{b}$	$.847^{c}$	$.331^{x}$	$.371^{xy}$	$.416^{y}$	$.404^{y}$
iso-butyric	$.020^{bc}$	$.027^{c}$	$.018^{b}$	$.018^{b}$	$.010^{a}$	.014	.017	.016	.016
iso-valeric	$.032^{a}$	$.057^{c}$	$.045^{b}$	$.038^{ab}$	$.030^{a}$	$.021^{x}$	$.026^{yz}$	$.028^{z}$	$.024^{xy}$
n-valeric	$.077^{a}$	$.134^{b}$	$.109^{b}$	$.117^{b}$	$.113^{b}$	.038	.063	.059	.048
Caloric equivalents (kcal)*									
Total VFA	1252.6^{a}	1470.0^{b}	1885.5^{c}	1732.2^{c}	1707.2^{c}	750.2^{x}	924.9^{yz}	1027.2^{z}	849.3^{xy}

*Based on heats of combustion given by Hodgman *et al.* (1960).
abcd Means on the same line with the same superscript letter do not differ significantly (P > .05), Cow A.
xyz " " " " " " " " " " " " " " (P > .05), Cow B.

The total moles of VFA in the rumens of the cows at six 2-hour intervals during the last 12 hours on their respective treatment regimens are illustrated graphically in Figure 4. The total quantities of VFA in the rumen of Cow A (lactating) when mineral salts were added to the drinking water were consistently higher than during the control period. The increases during the mineral regimens are partly attributable to the significantly higher concentrations of VFA in the rumen fluid and partly to the larger volumes of water present in the rumen. With Cow B (dry) the concentrations of VFA in the rumen fluid were not significantly different, hence the greater amounts of VFA found in the rumen on mineral regimens were more closely associated with substantial increases in the volume of water in the rumen (Table 20).

In general, with the exceptions noted above, these data suggest that mineral salts did not influence the production of one acid more than another, but rather that they had a similar effect either on the production or the absorption of all VFA.

The relative rates of VFA production (Table 22) were not significantly different ($P > .05$), except for n-valerate in Cow A. The individual results from which the means were calculated were variable, presumably owing to difficulties in sampling the in vitro fermentation flasks and to temperature fluctuations and other uncontrolled factors in the dairy barn during the course of these experiments. As the hay was in long form when fed, mixing of the rumen ingesta samples was very difficult and consequently, with time an important factor, the liquid samples obtained may not have been representative. If the differences in the rates of production are indicative of trends it would seem that the effects of minerals are to allow a greater overall production of VFA rather than to influence the production of individual fatty acids.

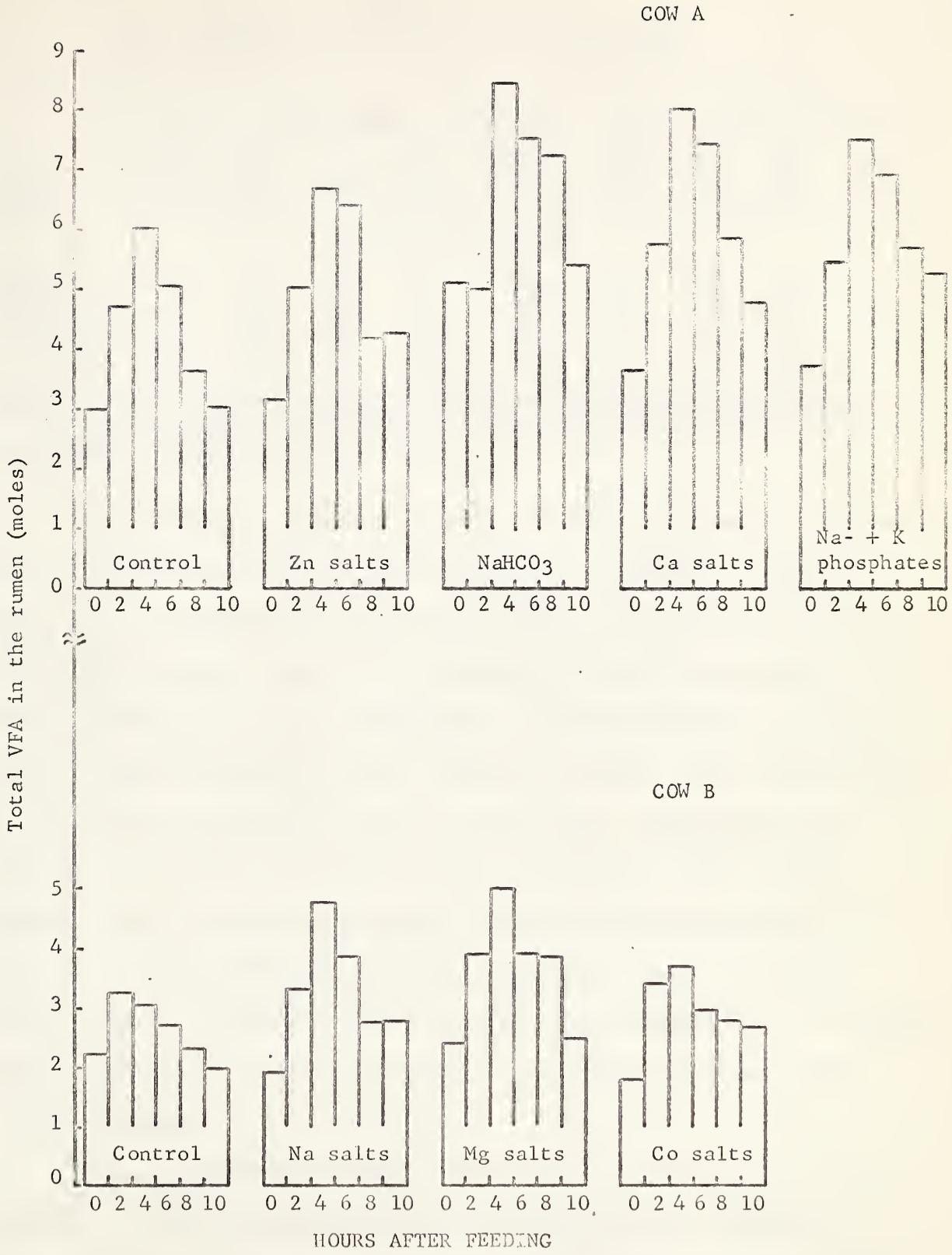

Figure 4. The effects of mineral salts in the water on the quantities of acids in the rumen at six times after feeding.

Table 22
Mean relative rates of VFA production in vitro

Acid	Cow A					Cow B			
	Control	Zn salts	$NaHCO_3$	Ca salts	Na- +K phosphates	Control	Na salts	Mg salts	Co salts
	(mmole/100 ml/30 min)								
acetate	.44	.77	.84	.58	.48	.54	.84	.61	.58
propionate	.15	.22	.29	.19	.16	.10	.15	.14	.11
n-butyrate	.16	.14	.20	.11	.14	.14	.18	.14	.12
n-valerate	0[a]	.04[b]	.04[b]	.04[b]	.03[b]	.02	.05	.03	.04

[ab]Within Cow A, means on the same line with the same superscript letter do not differ significantly ($P > .05$).

As previously discussed, minerals were associated with a decreased ratio of dry matter to liquid in the rumen. The consumption of water by Cow A was not altered when minerals were added to the water (Zn salts being the exception). From these observations it would seem that the addition of minerals in the drinking water elicited biochemical or physiological changes, presumably at the rumen wall tissues, which were reflected in the increased quantities of water in the rumen. Presumably either the transfer of water across the rumen wall or the flow of saliva would be involved; water transfer would appear to merit the most consideration as water is a freely diffusing substance moving from a lower to a higher osmotic pressure. Mineral salts in solution would contribute to the ruminal osmotic pressure and could, in this way, have a general systemic effect on rumen functions.

VFA are generally assumed to be absorbed from the rumen in direct proportion to their concentrations and at increasing rates under more acidic conditions. The average pH of the rumen ingesta was significantly lower ($P < .05$) when $NaHCO_3$, Ca salts and Na- + K phosphates or Na salts and Mg salts were added to the drinking water. Under the intra-rumen

environment associated with mineral salts in the drinking water (lower pH, higher VFA concentrations, a trend to an increased rate of production) one would assume that absorption of the VFA from the rumen would occur more readily than under the control rumen environment. The experimental work reported herein was not designed to provide a measure of the rates of absorption from the rumen - the total amounts of VFA absorbed were simply assumed to equal the total amounts of VFA produced.

In Cow B where the concentrations of VFA in rumen fluid were not significantly different between the control regimen and the Na and Mg regimens there appears to be no reason to doubt that if production of VFA was enhanced in the presence of mineral salts then absorption from the rumen was enhanced proportionately. In Cow A, consuming 9 lb more concentrate mixture per day than Cow B and lactating, it was found that the concentrations of VFA in rumen fluid on three of the mineral regimens (Table 21) were significantly higher ($P < .05$) than on the control regimen. $NaHCO_3$ at a level of 1420 ppm in the drinking water caused an average concentration of total VFA of 18.83 mmole/100 ml of rumen fluid versus 12.96 mmole/100 ml on the control regimen. Corresponding average pH values were 5.56 and 6.01 for the bicarbonate and control regimens respectively. The high concentrations of VFA and low pH values found under the bicarbonate regimen are suggestive of some interaction of the mineral salts on VFA absorption. Mineral salts (probably as electrolytes) may interfere with the absorption of VFA from the rumen, thus causing a buildup of VFA with a commensurate fall in ruminal pH. As these experiments were of a short term nature, further work is needed to refute or substantiate this possibility.

The average molar proportions of VFA in the rumen (Table 23) indicate that the mineral salts did not influence the molar percentages of VFA to any large degree. The lower percentages of acetate and n-butyrate commensurate with higher percentages of propionate and n-valerate observed

in vitro in the presence of Zn^{++} and Co^{++} were not observed *in vivo*. Zinc salts *in vivo* caused a slight decrease in the molar percentage of n-butyrate commensurate with a rise in the percentage of n-valerate. The small changes in the molar proportions of propionate and n-butyrate on the Na- + K phosphates regimen were of particular interest. The proportion of propionate was decreased and that of n-butyrate increased. The molar proportions of VFA in the rumen fluid at 2-hour intervals during the last 12-hour period of each test regimen are shown in Figures 5 and 6 for Cow A and B, respectively. As shown in Fig. 5 the molar percentages of propionate were higher and those of n-butyrate lower for the control, Zn salts, $NaHCO_3$ and Ca salts regimens than during the Na- + K phosphates regimen. In comparison with their controls, Zn salts, $NaHCO_3$, Ca salts, Na salts and Mg salts were associated with slightly depressed molar percentages of n-butyrate.

Table 23
Mean molar proportions of VFA in the rumen

	Cow A					Cow B			
	Control	Zn salts	$NaHCO_3$	Ca salts	Na- +K phosphates	Control	Na salts	Mg salts	Co salts
	(mole %)								
acetate	64.7	65.3	66.0	65.8	65.3	67.7	68.6	68.3	66.5
propionate	18.2	18.6	18.5	18.8	17.2	16.8	16.4	17.1	16.5
n-butyrate	14.0	11.6	12.8	12.5	14.8	12.8	11.7	11.7	14.0
iso-butyrate	.5	.6	.3	.3	.2	.5	.6	.5	.5
iso-valerate	.8	1.2	.7	.6	.5	.8	.8	.8	.9
n-valerate	1.8	2.7	1.7	2.0	2.0	1.4	1.9	1.6	1.6

In the lactating cow (Cow A) the following milk production and milk fat percentages were obtained:

		Test regimen				
		Control	Zn salts	$NaHCO_3$	Ca salts	Na- + K phosphates
Avg daily milk production	(lb)	23.0	21.0	22.0	23.2	20.0
Milk fat	(%)	4.72	4.40	4.86	4.83	5.38

It is interesting to note that a slight decrease vs. a marked increase in milk fat percentage was associated with the Zn salts vs. Na- + K phosphates. As noted above phosphate salts were associated with an increased ruminal molar proportion of n-butyric acid and a decreased molar proportion of propionic acid. Zinc salts were associated with a decreased proportion of n-butyrate and a rise in the molar percentage of n-valerate.

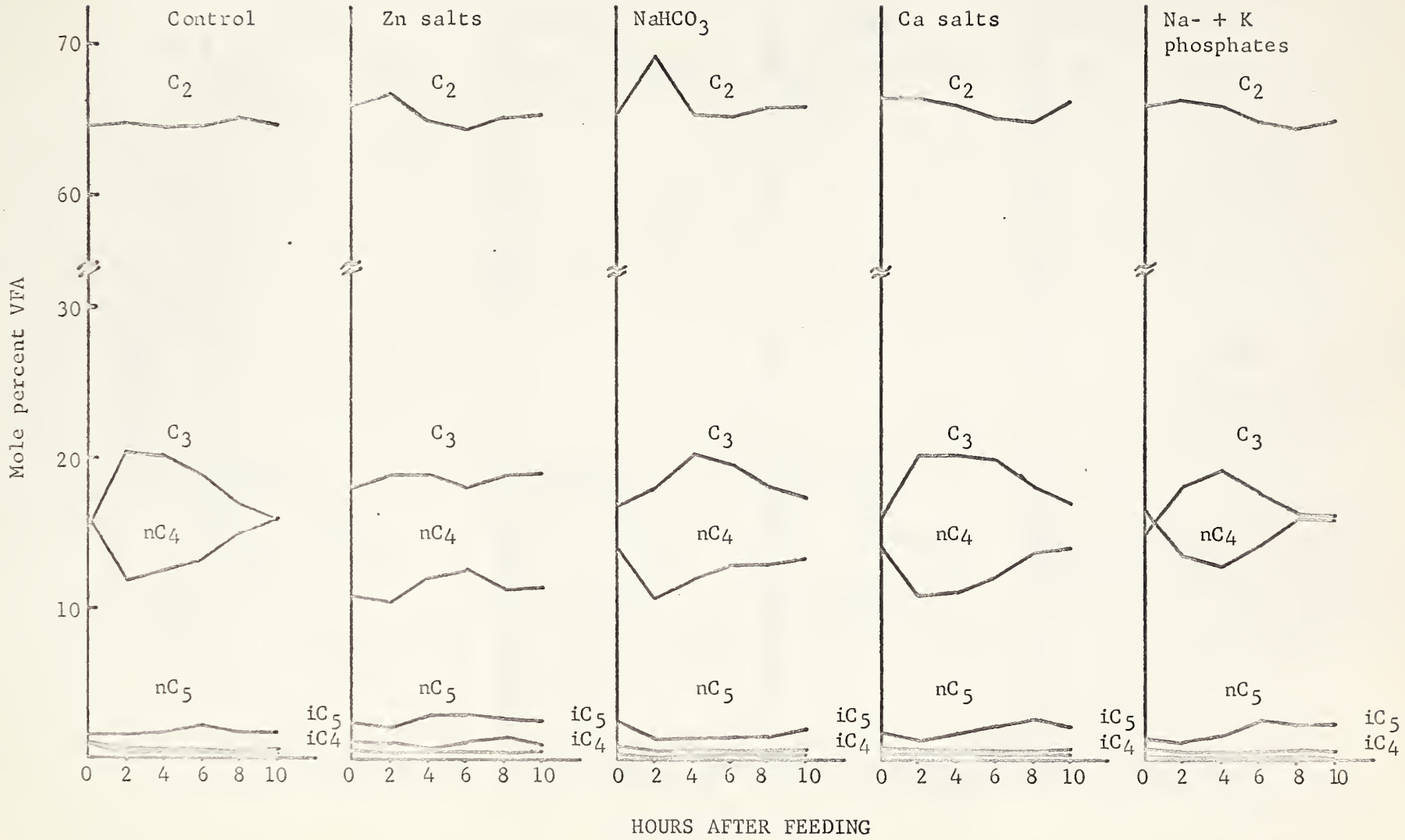

Figure 5. The effects of mineral salts in the water on molar proportions of VFA in the rumen fluid of Cow A. (C_2= acetate; C_3= propionate; nC_4= n-butyrate; nC_5= n-valerate; iC_5= iso-valerate; iC_4= iso-butyrate)

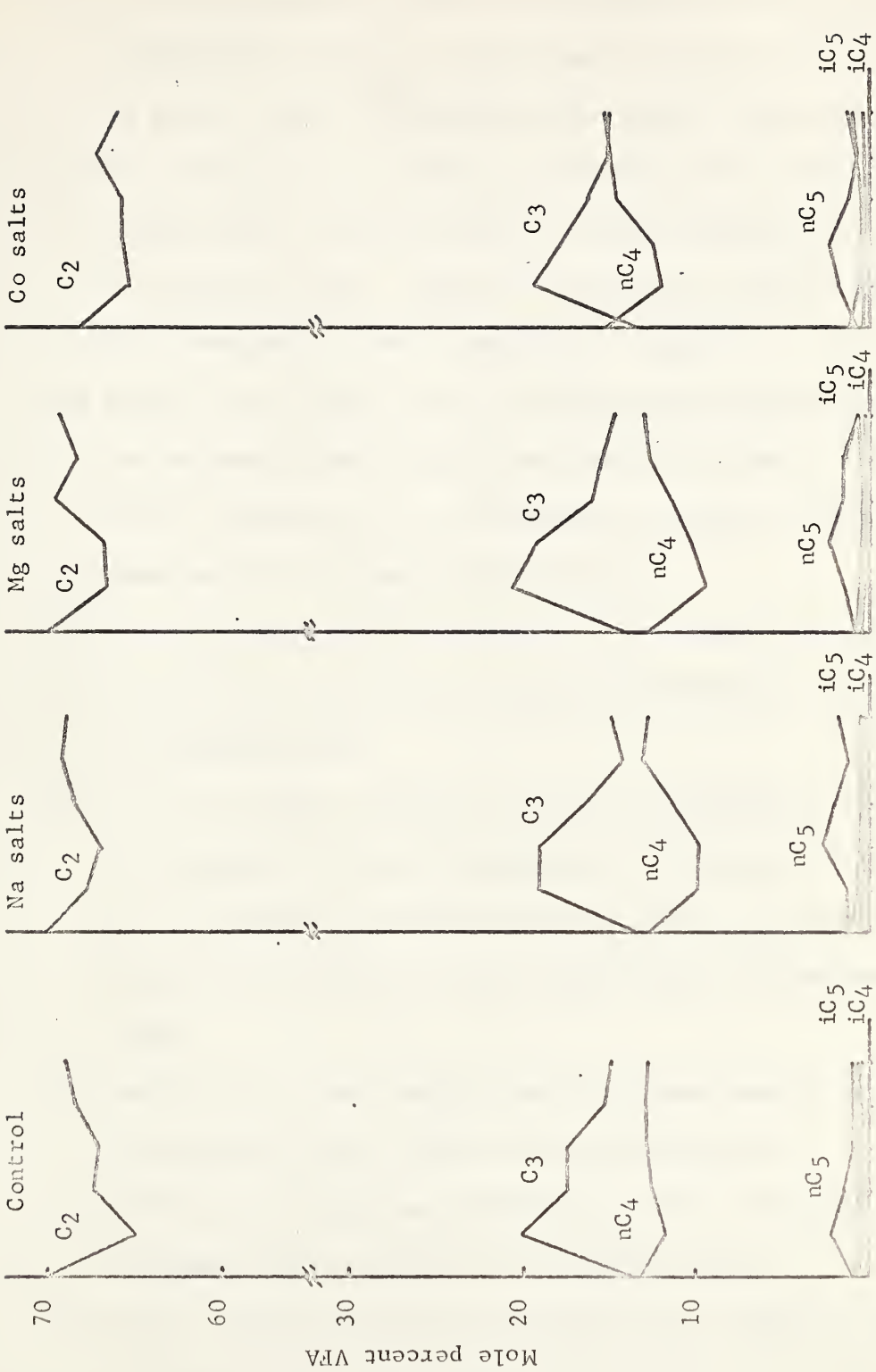

Figure 6. The effects of mineral salts in the water on molar proportions of VFA in rumen fluid of Cow B. (C_2=acetate; C_3=propionate; nC_4=n-butyrate; nC_5=n-valerate; iC_5=iso-valerate; iC_4=iso-butyrate)

Summary

(1) The concentrations of VFA in rumen fluid and the total quantities of VFA in the rumen of a lactating cow were increased when $NaHCO_3$, Ca salts, or Na + K phosphates were added to the drinking water.

(2) The concentrations of VFA in rumen fluid from a dry cow were not significantly increased when Na salts or Mg salts were added to the drinking water. The total quantities of VFA in the rumen were increased on the Na and Mg salt regimens.

(3) Mineral salts added to the drinking water caused increases in the average volume of water and number of moles of VFA in the rumen. A decrease in the percentage dry matter in the rumen was observed with all except the Zn salts.

(4) The relative rates of production of individual VFA were not altered significantly ($P > .05$) by the addition of minerals to the drinking water.

(5) Zn salts tended to depress the molar percentage of n-butyrate and increase the molar percentage of n-valerate in the rumen. Na- + K phosphates tended to depress the molar percentage of propionate and increase the molar percentage of n-butyrate in the rumen.

(6) The pH of the rumen ingesta was lower when mineral salts, other than Zn salts, were added to the drinking water.

(7) Milk fat percentage was increased with Na- + K phosphates and decreased with Zn salts in the drinking water.

(8) Water consumption by the lactating cow was unaltered when minerals, other than Zn salts, were added to the drinking water, whereas water consumption by the dry cow was substantially increased when Na and Mg salts were added to the water.

(9) Co salts caused a pronounced anorexia and Zn salts a mild anorexia when included in the drinking water at a concentration of 710 ppm.

GENERAL DISCUSSION

The results of *in vivo* and *in vitro* studies on the effects of mineral salts on production of VFA in the rumen and on percent fat in milk have shown that minerals do have an influence on the intra-rumen environment. This work suggests that, with some apparent exceptions, minerals probably exert their effects indirectly on VFA production rather than through direct action on the total microbial population or specific species thereof. Indirect influence would include the effects of mineral salts on water consumption, rumen dry matter content, rumen liquid volume, rumen pH and transport and utilization of the VFA.

The inclusion of mineral salts in the drinking water of fistulated dairy cows caused a decrease in the percent dry matter of the rumen contents. These results are in agreement with those reported by Nicholson *et al.* (1960, 1962), who found that the addition of alfalfa ash or 3% $NaHCO_3$ to the ration caused a lower percentage of dry matter in the rumen.

Increased water consumption comparable to that noted in the present studies with a natural well water and mineral salts in the drinking water has been reported previously for alfalfa ash (Nicholson *et al.*, 1960), $NaHCO_3$ (Cullision and Ward, 1961; Lassiter and Cook, 1961) and NaCl (Weeth *et al.*, 1960). The volume of water in the rumen of the cows when mineral salts were included in the water was increased and is in agreement with the results of Oltjen *et al.* (1962), who found that the volume of rumen fluid was increased with a basic mineral mixture in the ration.

The concentrations of VFA found *in vivo* when $NaHCO_3$, Ca salts and Na- + K phosphates were included in the drinking water of a lactating cow were significantly increased. When Na salts and Mg salts were added to the drinking water of the dry cow the concentrations of total VFA increased slightly but not significantly. While comparisons are confounded because

different minerals and only one-quarter as much concentrate mixture were included in the ration of the dry as of the lactating cow, it may be that the differences noted between the cows are attributable, at least in part, to physiological differences rather than simply mineral effects. It is not unrealistic to presuppose that the physiological activities and secretions of other organs in the cow exert profound influences on metabolism in the rumen. Emery and Brown (1961) found that depressed milk fat percentages were raised to normal values by feeding 1 lb Na- or $KHCO_3$ per cow per day. Unfortunately they did not study ruminal concentrations of VFA in their lactating cows, but, in contrast to the present results with a lactating cow, levels of total VFA in the rumens of dry cows were not altered by the addition of bicarbonates to the ration.

The levels of total VFA in the rumen were increased by feeding 5% alfalfa ash to fistulated steers and bulls (Nicholson *et al.*, 1960), by adding up to 3% $NaHCO_3$ to lamb rations (Raun *et al.*, 1962), by feeding a basic mineral mixture in a purified diet to steers (Oltjen *et al.*, 1962), and by including Na- and $KHCO_3$ in the purified diets of sheep (Van Campen and Matrone, 1960). On the other hand Nicholson *et al.* (1960) added 3% $NaHCO_3$ or 3% $NaHCO_3$ plus 2% limestone plus 1% K_2CO_3 to an all-concentrate ration fed to fistulated Jersey steers and decreased the level of total VFA in the rumen.

In the present studies the addition of mineral salts to the drinking water had, in most instances, no appreciable effects on the acetate to propionate ratios or on the molar proportions of other VFA in the rumen. Na- + K phosphates were associated with a slight decrease in the propionate to n-butyrate ratio. Bladen and Doetsch (1959), using washed bacterial cell suspensions from the rumen, found that variations in the concentration of the phosphate buffer used in the medium caused alterations in the ratios of VFA produced *in vitro*. Zn and Co salts were found to be toxic

at a level of 710 ppm in the drinking water. The markedly reduced acetate to propionate ratio observed in the *in vitro* work with Zn^{++} and Co^{++} was not demonstrated *in vivo*. The increased levels of propionate following the supplementation of pelleted rations with Co reported by Rhodes and Woods (1961, 1962) and Raun and Burroughs (1961) were not observed with Co salts in this work.

Except for a slight decrease in the n-butyrate to n-valerate ratio, Zn salts had no effect on the molar percentages of VFA found in the rumen. In contrast to the work of Bonomi and Cabassi (1960), the addition of Zn salts to the drinking water had a depressing effect on milk fat percentage. However, a mixture of three Zn salts was used at much higher levels than the $ZnSO_4$ fed by the Italian workers.

Commensurate with the increases found in the concentrations of VFA in the rumen fluid following addition of $NaHCO_3$, Ca salts, Na- + K phosphates, Na salts, or Mg salts to the drinking water were decreases in the rumen pH, indicating a more acid ingesta. The lowest pH values were associated with the highest levels of VFA in the rumen fluid which in turn were observed when 1420 ppm $NaHCO_3$ were added to the drinking water in the ration. These results are in contrast to other data which indicate that mineral salts tend to increase rumen pH (Nicholson *et al.*, 1960, 1962; Cullison and Ward, 1961; Raun *et al.*, 1962; Oltjen *et al.*, 1962; Emery and Brown, 1961).

Buffering mechanisms within the rumen apparently were affected by the dietary bicarbonate and to a lesser degree by several other mineral salts. Raun *et al.* (1962) noted that the buffering capacity of rumen fluid diminished with dietary $NaHCO_3$ supplementation, whereas Nicholson *et al.* (1962) found that $NaHCO_3$ and other buffer salts increased the buffering capacity of rumen fluid. The former workers have suggested that dietary bicarbonate probably reduced movement of rumen fluid buffering substances

into the rumen from the blood stream or peritoneal cavity.

The results obtained in this study, particularly with the lactating cow, on the effects of mineral salts on total VFA concentrations and pH of the rumen ingesta might be explainable on the basis of a mineral effect on the transport, absorption or metabolism of the VFA. Very little is known about the metabolic role or effect of different electrolytes in the rumen on VFA absorption or metabolism at the rumen wall. Emery and Brown (1961) suggested that the effect of Na- and $KHCO_3$ on the percent milk fat must be sought elsewhere than in changes in the molar proportions of VFA in the rumen. Oltjen et al. (1962) suggested that an acid rumen pH may not be optimum for the synthesis of B-vitamins. Unless sufficient amounts of B-vitamins are available maximal production and utilization of the VFA may not occur.

In the present experiment the consistent decrease in the percent dry matter in the rumen associated with all mineral salts tested, other than Zn salts, may be associated with a change in the rate of digestion of certain of the proximate principles of the ration. Balch and Johnson (1950) and Nicholson et al. (1960) found that the rate of breakdown of cellulose in the reticulo-rumen was closely related to the dry matter content of the ingesta; a low dry matter content favored the rapid breakdown of cellulose. Presumably the faster the rate of digestion the lower would be the total dry matter in the rumen.

The increased digestibility noted when alfalfa hay, or alfalfa ash, was added to the rations containing a high percentage of corn cobs or poor quality roughage may be attributable in part to an increased volume of water in the rumen associated with increased ash intake, rather than entirely to improved digestibility in the presence of trace minerals in the ash as suggested by Barnett and Reid (1961). However, supplemental minerals have not been shown to significantly affect digestibility when an all-concentrate

ration (Nicholson et al., 1962), a pelleted ration (Lassiter and Cook, 1961) or a number of common lactating dairy cattle rations (Chance and Loosli, 1961) were fed.

In the present work a tendency was noted for the rates of VFA production to be increased during mineral salt supplementation. Since the rumen dry matter percentages were lower during the mineral supplementation it follows that the rate of digestion may have been increased as well. However, it is unlikely that the increase in the rate of digestion that may have occurred could account in full for the increased concentrations of VFA in the rumen fluid. The premise that the mineral salts may have tended to interfere with the absorption and transport of VFA from the rumen would seem to offer a more probable explanation for the increased levels of VFA found in the rumen. Further, major physiological activities, e.g. lactation, growth, etc., may exert profound influences on the production and/or absorption of VFA, particularly under conditions of high mineral salt intake which modify the water balance of the rumen.

GENERAL SUMMARY

I. A simple quantitative technique was developed, utilizing gas-liquid chromatography, for the direct analysis of VFA in acidified rumen fluid. Water solutions of the VFA were separated with a column of 10 percent 12-hydroxystearic acid on Fluoropak 80. With helium as the carrier gas and a flame ionization detector, acetic, propionic, n-butyric, iso-butyric, iso-valeric and n-valeric acids were measured quantitatively in amounts as low as 0.01 mmole/100 ml of rumen fluid.

II. A natural well water of high soda content, a water high in Na- and $KHCO_3$, a water high in Na_2- and K_2CO_3, and demineralized water were fed to lactating Jersey cows. The test waters had no measurable effect on the percentage fat in the milk. The addition of an oat-barley silage to the rations containing the test waters had no apparent effect on the percent fat in the milk.

The natural well water decreased the acetate to propionate ratio. Silage plus the natural water induced a decrease in the molar proportion of propionate and an increase in the n-butyrate percentage. The test water high in Na_2- and K_2CO_3 was associated with a molar ratio shift from n-butyric to propionic acid. Neither the water containing high levels of Na- and $KHCO_3$ nor the demineralized water were associated with any consistent changes in the molar ratios of the VFA.

III. Several cations, anions and miscellaneous mineral salts were studied for their effects on *in vitro* rumen fermentations. Zn^{++} and Co^{++} caused a depression in the production of acetic and n-butyric acids commensurate with an increase in the production of propionic and n-valeric acids. CO_3^{--}, particularly in the presence of Zn^{++} and Co^{++}, was associated with a lower propionic acid production. Na- or $KHCO_3$ had no effect on VFA production

in vitro. NO_3^- caused a depression of total VFA production with a shift in the relative proportions of VFA from acetic and n-butyric acids to propionic and n-valeric acids.

Eight natural well waters of high mineral salts content were screened by the *in vitro* rumen fermentation procedure. Considerable variation was associated with the different waters and the results obtained with the mineral waters and distilled water were not significantly different ($P > .05$) although the relative proportion of n-butyric acid tended to be higher with distilled water than with the natural waters.

IV. The effects of several mineral salts on ruminal VFA and intra-rumen environment were studied *in vivo* using fistulated dairy cows. The concentrations of VFA in rumen fluid were markedly increased in a lactating cow consuming water containing $NaHCO_3$, Ca salts or Na- + K phosphates. The concentrations of VFA in rumen fluid were increased, but not significantly, when Na or Mg salts were added to the drinking water of a dry cow. Mineral salts, with the exception of Zn salts, were associated with an increased volume of water, a lower percent dry matter, greater quantities of VFA, and a lower pH in the rumen. The relative rates of VFA production tended to be greater in the presence of minerals. The Na- + K phosphate treatment was associated with a reduced molar ratio of propionate to n-butyrate. Zn salts tended to depress the molar ratio of n-butyrate to n-valerate, however the marked depression of the acetate to propionate ratio observed *in vitro* was not observed *in vivo*. In general the other mineral salts had no appreciable effect on the molar ratios of VFA in the rumen. At a level of 710 ppm Zn or Co salts caused anorexia in the cattle.

Water consumption was increased by the dry cow when Na or Mg salts were included in the drinking water. The water consumption of the lactating cow was not altered appreciably by the addition of $NaHCO_3$, Ca salts or

phosphate salts to the drinking water. Percent milk fat was slightly reduced on the Zn regimen and substantially increased on the phosphates regimen.

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Appendix Table A
Concentrate mixture used in part III (*in vitro*) and part IV (*in vivo*)

Ingredients	lb
Oats (crushed)	780
Barley (crushed)	780
Soybean oil meal (44%)	300
Dehydrated alfalfa meal	100
NaCl	20
Bone meal	10
Limestone	10
Ethylenediamine dihydriodide* (20 g/oz)	2
Total	2002

*Hi-Amine, Allied Laboratories,(Canada) Limited, Don Mills, Ontario.

Appendix Table B
Mean squares (Table 12), the effect of cations, anions and trials on VFA production *in vitro*

Source of variation	df	Total VFA	acetic acid	propionic acid	n-butyric acid	iso-butyric acid	iso-valeric acid	n-valeric acid
Total	35							
Cation	5	3.6301**	4.8356**	1.5127**	.7130**	.0003	.0021	.0511**
Anion	2	.0305	.1323	.2901*	.0114	0	.0005	.0001
Trial	1	44.5557**	19.4334**	.9152**	1.1485**	.0066**	.0034	.0097
Cation x anion	10	.1602	.1247	.1023	.0599**	.0001	.0003	.0028
Cation x trial	5	.6714*	.1761	.0885	.0719**	.0001	.0005	.0025
Anion x trial	2	.8308*	.1639	.2060*	.0136	.0002	0	.0033
Residual	10	.1588	.0610	.0417	.0066	.0001	.0012	.0078

*Significant at 0.05 level of probability
**Significant at 0.01 level of probability

Appendix Table C
The production of VFA in vitro, 6 x 3 block experiment, trial 1

	Anion	Cation					
		Na^+	K^+	Ca^{++}	Mg^{++}	Zn^{++}	Co^{++}
		(mmole/100 ml/24 hour)					
Total VFA	Cl^-	10.84	11.39	10.58	10.76	8.62	10.54
	CO_3^{--}	10.69	10.96	10.57	10.52	8.89	10.46
	SO_4^{--}	11.46	11.50	11.39	11.28	9.03	10.14
acetic acid	Cl^-	6.35	6.76	6.15	6.35	4.30	4.83
	CO_3^{--}	6.61	6.51	6.20	6.11	4.90	5.83
	SO_4^{--}	6.81	6.83	6.83	6.72	4.46	4.59
propionic acid	Cl^-	2.62	2.79	2.58	2.61	3.38	4.46
	CO_3^{--}	2.32	2.52	2.60	2.53	2.90	3.02
	SO_4^{--}	2.76	2.78	2.56	2.63	3.62	4.32
n-butyric acid	Cl^-	1.58	1.51	1.57	1.53	.55	.63
	CO_3^{--}	1.43	1.61	1.51	1.57	.65	1.26
	SO_4^{--}	1.58	1.59	1.61	1.60	.60	.65
iso-butyric acid	Cl^-	.04	.05	.04	.05	.03	.04
	CO_3^{--}	.04	.05	.03	.04	.03	.02
	SO_4^{--}	.04	.04	.06	.04	.04	.04
iso-valeric acid	Cl^-	.09	.11	.07	.05	.06	.12
	CO_3^{--}	.09	.11	.07	.07	.06	.05
	SO_4^{--}	.07	.09	.09	.11	.03	.04
n-valeric acid	Cl^-	.16	.17	.17	.17	.30	.46
	CO_3^{--}	.20	.16	.16	.20	.35	.28
	SO_4^{--}	.20	.17	.24	.18	.28	.50

Appendix Table D
The production of VFA *in vitro*, 6 x 3 block experiment, trial 2

	Anion	Cation					
		Na^{+}	K^{+}	Ca^{++}	Mg^{++}	Zn^{++}	Co^{++}
		(mmole/100 ml/24 hour)					
Total VFA	Cl^{-}	9.96	8.27	8.45	8.60	8.26	7.48
	CO_3^{--}	9.56	8.12	9.01	8.52	6.98	8.55
	SO_4^{--}	9.19	7.69	9.01	7.89	6.38	7.65
acetic acid	Cl^{-}	5.59	4.75	4.98	4.89	3.33	3.43
	CO_3^{--}	5.73	4.72	5.12	5.10	3.20	4.02
	SO_4^{--}	5.32	4.45	5.31	4.49	2.85	3.41
propionic acid	Cl^{-}	2.72	2.38	2.20	2.26	3.73	3.33
	CO_3^{--}	2.50	2.35	2.39	2.34	2.98	2.94
	SO_4^{--}	2.43	2.02	2.25	2.14	2.86	3.44
n-butyric acid	Cl^{-}	1.34	.96	1.05	1.15	.69	.50
	CO_3^{--}	1.08	.82	1.20	.88	.52	1.10
	SO_4^{--}	1.21	1.05	1.20	1.01	.42	.42
iso-butyric acid	Cl^{-}	.03	0	0	.03	0	0
	CO_3^{--}	.04	.01	.02	.01	0	.01
	SO_4^{--}	.01	.03	.02	.02	0	0
iso-valeric acid	Cl^{-}	.06	.08	.07	.14	.01	.02
	CO_3^{--}	.05	.08	.09	.06	.03	.04
	SO_4^{--}	.06	.03	.07	.05	.01	.08
n-valeric acid	Cl^{-}	.22	.10	.15	.13	.50	.20
	CO_3^{--}	.16	.14	.19	.13	.25	.44
	SO_4^{--}	.16	.11	.16	.18	.24	.30

Appendix Table E
Mean squares (Table 15), effect of natural well waters, substrates, Na_2CO_3 and trials on VFA production and pH *in vitro*

Source of variation	df	Total VFA	acetic acid	propionic acid	n-butyric acid	iso-butyric acid	iso-valeric acid	n-valeric acid	24-hour pH
Total	71								
Water	8	1.4109	.4282	.0958	.0821**	.0007**	.0020	.0042	.1808**
Substrate	1	.6962	.6825	.7260	.5513**	.0008*	.0025	0	.1840**
Na_2CO_3	1	2.1563	3.8596**	.0039	.3254**	.0003	.0003	.0006	7.3600**
Trial	1	4.8985	4.6563**	.0028	.1663**	.0006*	.0221**	.0317**	8.8620**
Water x substrate	8	.2840	.1079	.1335	..0078	.0001	.0017	.0016	.0040
" x Na_2CO_3	8	2.9688*	1.1307*	.3292	.0215	.0003	.0024	.0010	.0064
" x trial	8	.6547	.3027	.0947	.0461**	.0003	.0011	.0059	.0752**
Substrate x Na_2CO_3	1	.5408	.0292	.3946	.0031	0	.0012	.0014	.0004
" x trial	1	.0200	.4951	.0087	.5442**	.0001	.0019	.0030	.0002
Na_2CO_3 x trial	1	85.1512**	36.2526**	5.1574**	.4232**	.0092**	.0060*	.0105	.0134
Residual	33	1.2381	.3853	.2632	.0136	.0001	.0013	.0031	.0066

*Significant at 0.05 level of probability
**Significant at 0.01 level of probability

Appendix Table F
Mean squares, effect of natural well waters concentrated five-fold, substrates and Na_2CO_3 on VFA production and pH *in vitro*

Source of variation	df	Total VFA	acetic acid	propionic acid	n-butyric acid	iso-butyric acid	iso-valeric acid	n-valeric acid	24-hour pH
Total	35								
Water	8	.9646	.3073	.1469	.0885	.0002	.0006	.0069	.2367**
Substrate	1	.4761	1.1700	.4466	1.0955**	.0007	.0001	.0014	.0982**
Na_2CO_3	1	30.1035**	8.2274**	2.4388	.7453**	.0031**	.0045*	.0030	4.0000**
Water x substrate	8	.5747	.1653	.3127	.0142	.0001	.0002	.0028	.0011
" x Na_2CO_3	8	4.4577	1.8216	.5528	.0220	.0002	.0010	.0041	.0133*
Substrate x Na_2CO_3	1	.0400	.0103	.0890	.0082	0	0	.0079	.0121
Residual	8	2.5140	.5963	.5188	.0332	.0001	.0004	.0038	.0026

*Significant at 0.05 level of probability
**Significant at 0.01 level of probability

Appendix Table C
Mean squares (Table 20), volume of water, percent dry matter and pH of rumen contents

Source of variation	Ruminal criteria					
	df	Volume of water	df	Percent dry matter	df	pH
Cow A						
Treatment	4	17.0877**	4	3.9450**	4	.3589**
Time	2	313.5983**	2	.1250	5	.3340**
Residual	8	1.6093	8	.0913	20	.0466
Cow B						
Treatment	3	33.4227*	3	7.0033**	3	.0559**
Time	2	105.7515**	2	1.3500*	5	.0598**
Residual	6	6.4336	6	.1267	15	.0087

*Significant at 0.05 level of probability
**Significant at 0.01 level of probability

Appendix Table H
Mean squares (Table 21), concentrations, total quantities and caloric equivalents of VFA in the rumen

Source of variation	df	Total VFA	acetic acid	propionic acid	n-butyric acid	iso-butyric acid	iso-valeric acid	n-valeric acid	caloric equivalent of VFA
Cow A									
				Concentration (mmoles/100 ml rumen fluid)					
Treatment	4	38.4614**	17.7032**	1.3135**	.9414**	.0016**	.0039**	.0098	
Time	5	20.7076**	7.9075**	1.3281**	.3529**	.0006	.0006	.0239**	
Residual	20	2.3083	.8872	.1069	.0551	.0002	.0004	.0046	
				Quantities (moles in rumen)					(kcal/1000)
Treatment	4	4.6656**	2.1760**	.1664**	.0970**	.0002**	.0007**	.0026**	.3714**
Time	5	8.9750**	3.7098**	.4699**	.1035**	.0001*	.0001	.0052**	.7566**
Residual	20	.2571	.0976	.0136	.0057	.00002	.0001	.0005	.0238
Cow B									
				Concentration (mmoles/100 ml rumen fluid)					
Treatment	3	3.4175	2.2154*	.2510**	.0019	.0001	.0001	.0045	
Time	5	7.0241**	2.5553**	.7526**	.0401	.0002	.0005**	.0143**	
Residual	15	1.0475	.4256	.0195	.0184	.0001	.0001	.0019	
				Quantities (moles in rumen)					(kcal/1000)
Treatment	3	1.0960**	.5698**	.0375**	.0086*	.00001	.00005*	.0008	.0824**
Time	5	2.1551**	.8774**	.1385**	.0163**	.00003	.00002	.0024**	.1809**
Residual	15	.1610	.0705	.0068	.0019	.00001	.00001	.0003	.0138

*Significant at 0.05 level of probability
**Significant at 0.01 level of probability